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# Quantification of the Human Granulocytic Ehrlichiosis Agent Based on Analysis of rRNA Isolated from Control and Infected HL-60 Cells

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**Human granulocytic ehrlichiosis (HGE) is an emerging vector-borne disease caused by an *Ehrlichia* species similar or identical to *E. equi* and *E. phagocytophila*. Previous studies have shown that the pathogen can be cultivated *in vitro* in permissive cells such as human promyelocytic HL-60 leukemia cells. The mechanism(s) of its infection and propagation in target cells, however, is not well understood, due in part to lack of a method capable of quantitatively determining the amount of the infectious agent. Although several assays currently exist for the HGE agent, they are mostly qualitative and have a number of limitations. In this report, size differences between prokaryotic and eukaryotic rRNAs are utilized to quantitatively assay the HGE agent in HL-60 cells. By comparing the integrated intensity of agarose gel resolved HGE-specific rRNA in host cells, with identically prepared and analyzed rRNA isolated from known quantities of *E. coli* (JM 109), it is possible to calculate the *E. coli*-equivalence of the HGE agent present in HL-60 cells according to the equation: Y (*E. coli*, in viable cells × $10^8$) = −2.573 + 0.11X (% infection by the HGE agent in HL-60 cells). The method described is reproducible, sensitive, and is not limited by availability of antisera. Furthermore, since the assay has no designer primer and repeated amplification requirements, it can be easily disseminated to and standardized in other laboratories.** 

Human granulocytic ehrlichiosis (HGE) is a carrier-transmitted disease, which was first described in Minnesota and Wisconsin in 1994, and more recently also in several other localities across the United States and in Europe (1–10). HGE is caused by intracellular, gram-negative Ehrlichia that multiply primarily in granulocytes. Sequence analysis of the 16S rRNA gene suggests that the HGE infectious agent is closely related to *E. equi* and *E. phagocytophila* (11). Like *Borrelia burgdorferi*, the causative bacterium of Lyme disease, the HGE agent is transmitted by *Ixodes scapularis* ticks. Transmission of both infectious agents by the same reservoir host has been reported in humans (12, 13). The infection produces a multi-symptom illness that usually requires only brief hospitalization. In rare cases, however, HGE has been known to cause fatalities. This is probably due to confounding factors, including delay and/or error in diagnosis and treatment.

Several assays currently exist for identifying the HGE agent. These include (i) detection of inclusion bodies (morulae) in neutrophils of suspected cases (12, 14), (ii) immunofluorescence assay (IFA) that measures the reactivity of acute or convalescent sera from patients against *E. equi* antigen isolated from HGE agent-infected horses (12, 14, 15). Alternatively HGE antigens derived from infected HL-60 cells can be detected using patient sera by enzymatic immunoassay (EIA) (16, 17), (iii) PCR using primers targeted against the spacer region of the rRNA gene (11, 12, 14), and (iv) culture-positivity in receptive cells such as HL-60 leukemia cells (12, 14, 17–23). These assays may introduce false positive results contributing to errors in diagnosis of the disease. In addition, none of the HGE detection methods currently used in clinical settings allow the quantification of bacterial load. This would clearly be advantageous for (i) administering and monitoring the early efficacy control of the therapeutic regimen, (ii) correlative analysis of the clinical presentations of HGE and bacterial burden, and (iii) the study of the influence of immune surveillance mechanisms on propagation and elimination of the HGE agent in animal models. In this communication, size differential existing between prokaryotic and eukaryotic rRNA is explored to provide a basis for developing a quantitative method to evaluate the presence of the HGE agent

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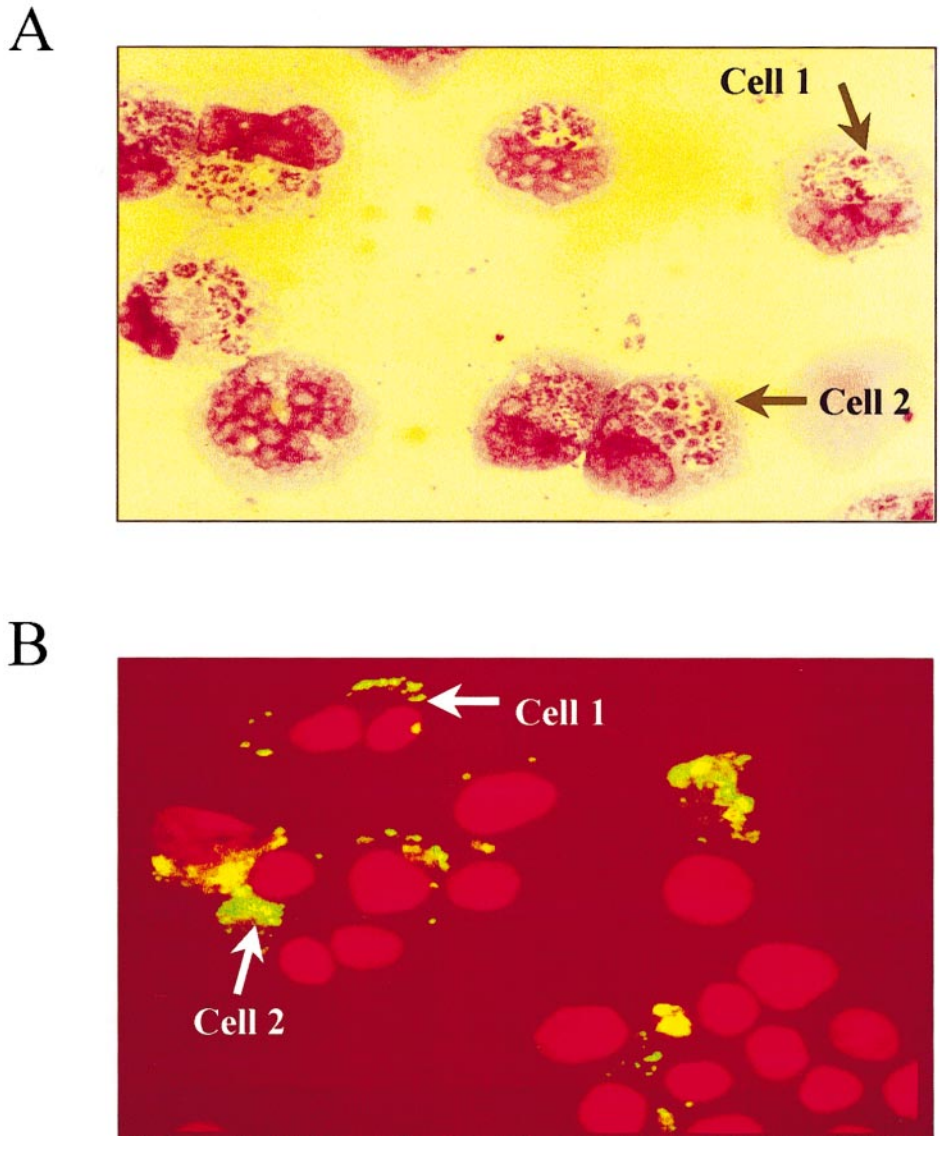


**FIG. 1.** Presence of the HGE agent based on presence of morulae (A) and IFA (B). (A) Photomicrograph of Wright's stain showing sparsely (cell 1) and densely populated morulae (cell 2) in HL-60 cells infected by the HGE agent. (B) IFA analysis of infection of HL-60 cells by the HGE agent. HGE-infected HL-60 cells are incubated with FITC-conjugated convalescent sera from patients diagnosed with HGE, and counter-stained with propidium iodide to reveal nuclei of host HL-60 cells. Cells 1 and 2 show respectively intensely and lightly stained areas using the IFA method.

in HL-60 cells. Specifically, the intensity of HGE-specific rRNA, revealed by ethidium bromide staining of agarose gel-resolved RNA isolated from control and infected HL-60 cells, is compared with intensity of rRNA prepared from known quantities of *E. coli*, to calculate the *E. coli*-equivalence of the HGE agent present in HL-60 cells.

## MATERIALS AND METHODS

*Isolation and culture of New York (NY) HGE isolates.* The New York HGE isolates 7030 and 7034 were established from suspected cases, as described previously (12, 19, 20). Infected cells were propagated in HL-60 cells using RPMI-1640 without antibiotics and supplemented with 10% heat-inactivated fetal bovine serum (FBS). Culture positivity in HL-60 cells and percent of infection was based on morulae in slide preparations, as well as by IFA using FITC-conjugated human sera obtained from the convalescent phase of patients infected with the HGE agent (12, 20). Routinely cytospin preparations containing 200 cells were evaluated for the presence of the HGE agent and determination of percent of infection.

*Propagation of bacterial cultures HB101 and JM109. E. coli* strains HB101 and JM109 were purchased from Promega Corp. (Madison, Wisconsin), or provided by Dr. M. Lee (JM 109) of this Department. Bacterial cultures were propagated in sterile LB broth

**TABLE 1**

Comparison of Four Existing Methods to Demonstrate the Presence of the HGE Agent

| Method | Assay |
|---|---|
| One | Presence of morulae by Wright's stain |
| Two | Seroconversion with *E. equi* antigen or HGE agent antigen |
| Three | PCR using 16S rRNA specific primers |
| Four | Culture positivity in HL-60 cells |

at 37°C in a rotary shaker. Growth was monitored by measuring $OD_{600}$. The quantity of viable bacterial cells was calculated on the basis of 1 $OD_{600} = 8 \times 10^8$ viable cells.

*Isolation and analysis of RNA from cultured bacteria and control/HGE agent-infected HL-60 cells.* Total RNA was isolated from *E. coli* and HL-60 cells using the RNeasy kit purchased from Qiagen, Inc. (Valencia, California), as follows. *E. coli* in log-phase growth ($1–3 \pm 10^9$ cells) were pelleted by a 3–5-min centrifugation at 5,000 xg. The pellets were resuspended in 100 (l buffer containing lysozyme (stock, 2.8 mg/ml), as detailed in the protocol provided by Qiagen. RNA recovery from $5 \times 10^8$ cells were $17.8 \pm 0.2$ (n = 3) for HB101, $15.6 \pm 1.8$ (n = 3) for JM109 from Promega and $17.0 \pm 2.2$ (n = 9) for JM109 from Dr. M. Lee. The same procedure was used to isolate RNA from control and HGE agent-infected HL-60 cells. Varying amounts of bacterial and HL-60 RNA were denatured with glyoxal, separated by electrophoresis on 1 or 1.4% agarose gels, stained with ethidium bromide, visualized under a UV source, and analyzed using a digital imaging system from Alpha Innotech Corporation.

## RESULTS AND DISCUSSION

*Technical issues and limitations in diagnosis of the HGE agent.* Table 1 lists the four methods frequently used to detect the presence of the HGE agent. The most common assay (method one) is based on cytoplasmic inclusions, referred to as morulae, present in granulocytes or neutrophils (12, 15, 22). Limiting features of this method are its qualitative and subjective nature. Thus, as illustrated in Fig. 1A, sparcely or densely populated morulae in a given cell are assigned equal weights in calculating the percentage of infection, based on currently accepted criteria. Also, clusters of morulae inside HL-60 cells at times could be mistakenly identified as granules and vice versa, thereby giving inaccurate estimation of infection. Another drawback of method one is that a negative smear result does not necessarily eliminate infection by the HGE agent. In method two, which relies on reactivity of human sera with *E. equi* antigens or with HGE antigens, false positives can result from Rocky mountain spotted fever, Q fever, murine typhus, Lyme disease (24, 25). Another potential deficiency of this method is that no standards exist for relating IFA data to different load of the HGE agent. Thus, intensely staining IFA spots are scored equally as less intense IFA areas (Fig. 1B). In addition, positive serological testing results only indicate prior exposure and not active infection. In molecular probe-based diagnosis of the HGE agent (method three), false positives, as well as variations in the design and selection of primers between laboratories, underscore some of its intrinsic limitations (11, 14, 26–29). The most reliable method for unequivocal demonstration of active HGE agent is culture positivity in permissive cells, e.g., HL-60 cells (method four). This method has drawbacks of cost, requirement for tissue culture expertise, as well as inter-isolate variability, uncertainty and ambiguity on time required before to culture positivity can be definitively established. Also noteworthy is the fact that none of the methods described provide a reasonable and objective approximation of the HGE agent load at the time of infection or upon propagation *in vitro*.

*Different propagation patterns of the HGE agent in HL-60 cells.* Since 1996, this laboratory has been involved in studies designed to establish HGE isolates from suspected cases, testing in total 191 blood specimens (92 in 1996, 53 in 1997 and 46 in 1998). At least three propagation patterns have been identified for the

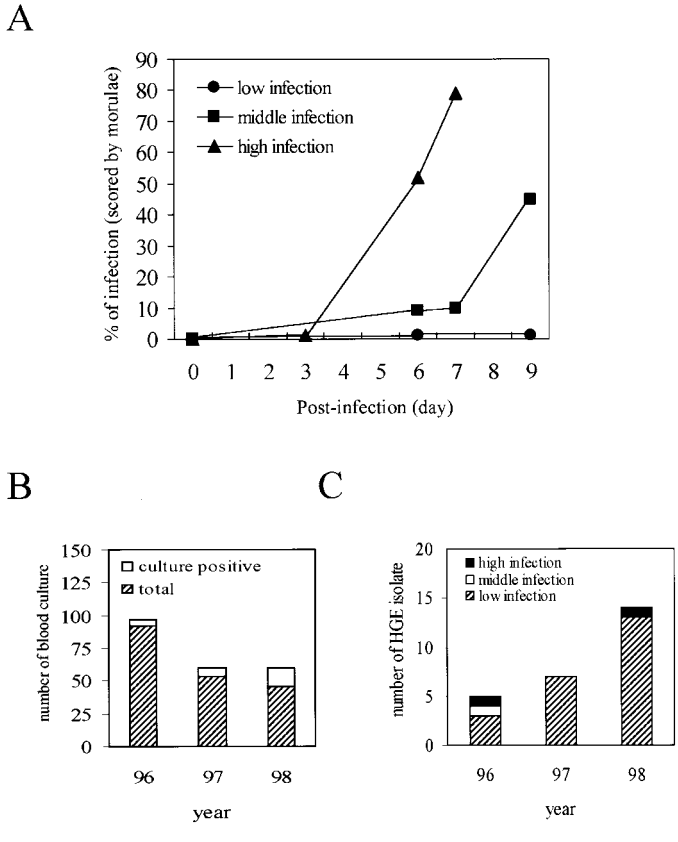


**FIG. 2.** Comparison of HGE agent propagation characteristics in HL-60 cells. (A) Suspected HGE blood specimens tested in 1996–1998. (B) Variations in propagation pattern of the HGE agents in HL-60 cells. (C) Distribution of various HGE agent propagation patterns observed in culture-positive isolates in 1996–1998.

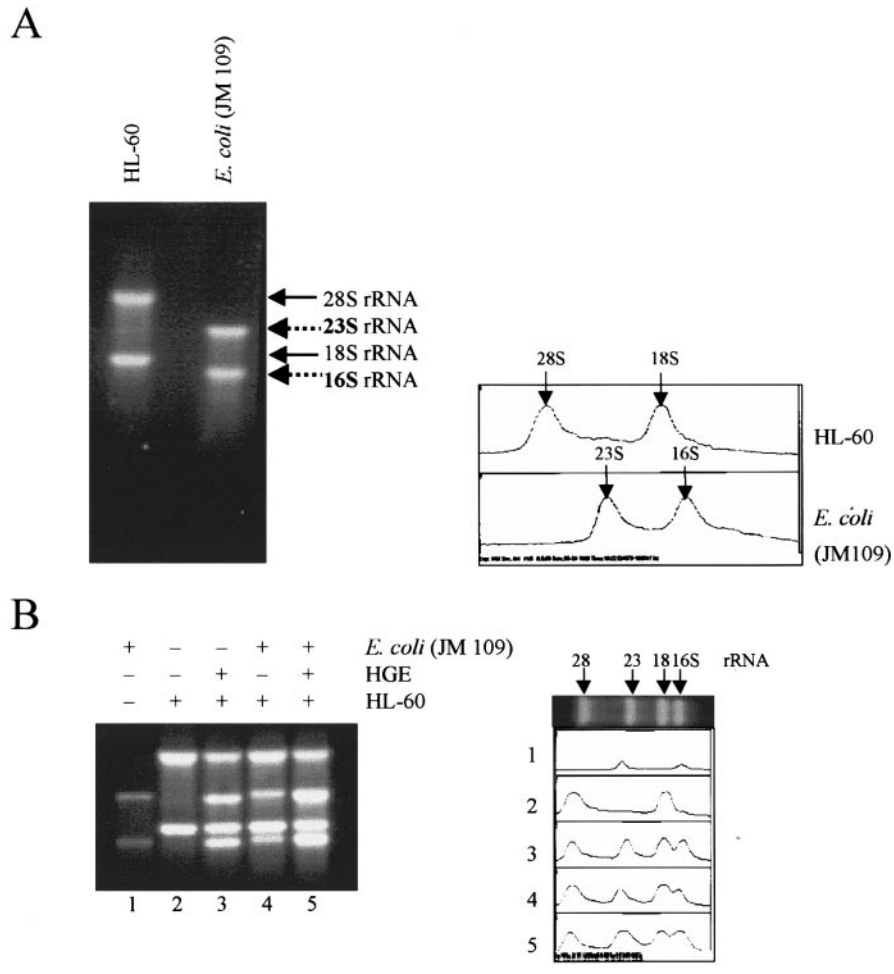


**FIG. 3.** Appearance of HGE-specific rRNA in HL-60 cells. (A) Photomicrograph of ethidium bromide stains of rRNA isolated from uninfected HL-60 and *E. coli* JM109 cells. The adjoining panel shows analysis of rRNA using a digital imaging system from Alpha Innotech Corporation. (B) Photomicrograph showing rRNA isolated from *E. coli* JM109 cells and from HGE agent infected HL-60 cells. Lane 1, rRNA isolated from *E. coli* JM109 cells; lane 2, rRNA isolated from uninfected HL-60 cells; lane 3, rRNA isolated from HL-60 cells combined with *E. coli* JM109 cells; lane 4, rRNA isolated from HL-60 cells infected with HGE isolate NY 7030; lane 5, rRNA isolated from HL-60 cells infected with HGE isolate NY 7030 combined with *E. coli* JM109 cells. The gel was also analyzed using a digital imaging system from Alpha Innotech Corporation. Results of such an analysis are shown in the adjoining panel.

HGE agent in HL-60 cells (Fig. 2A). In pattern 1, high infection (≥50–60% infection) is rapidly established, within 1 week, in host cells. By comparison, pattern 2 isolates typically result in 10% host infected in 1 week, with evidence for significant infection (≥40%) requiring considerably longer periods. In a third category of isolates (pattern 3), infection remains low at 2–5%, even after months in culture. Figure 2B compares the success rate in number of HGE isolates confirmed in consecutive years, between 1996–98. Figure 2C shows the distribution of isolates with the three described propagation patterns for isolates established in 1996–98. The bases for these different propagation patterns observed have not been elucidated but could relate to variations in the bacterial load at clinical presentation, which in turn significantly affect pathogen-host interaction, and give rise to a gradient of cellular anomaly that accounts for the perpetuation behavior of the pathogen in the host. To test such a possibility, a method must be developed that quantifies the HGE agent. Ideally, for the method to be generally applicable and easily disseminated to other laboratories, it should utilize features that relate HGE agent to a ubiquitous and commercially available reference standard.

*Appearance of HGE-specific rRNA in RNA isolated from HGE infected HL-60 cells.* The inception of the central dogma concept provides the basis for the long established role of RNAs as a family of molecules serving as important intermediates in gene expression, by acting both as a repository and as essential carriers of

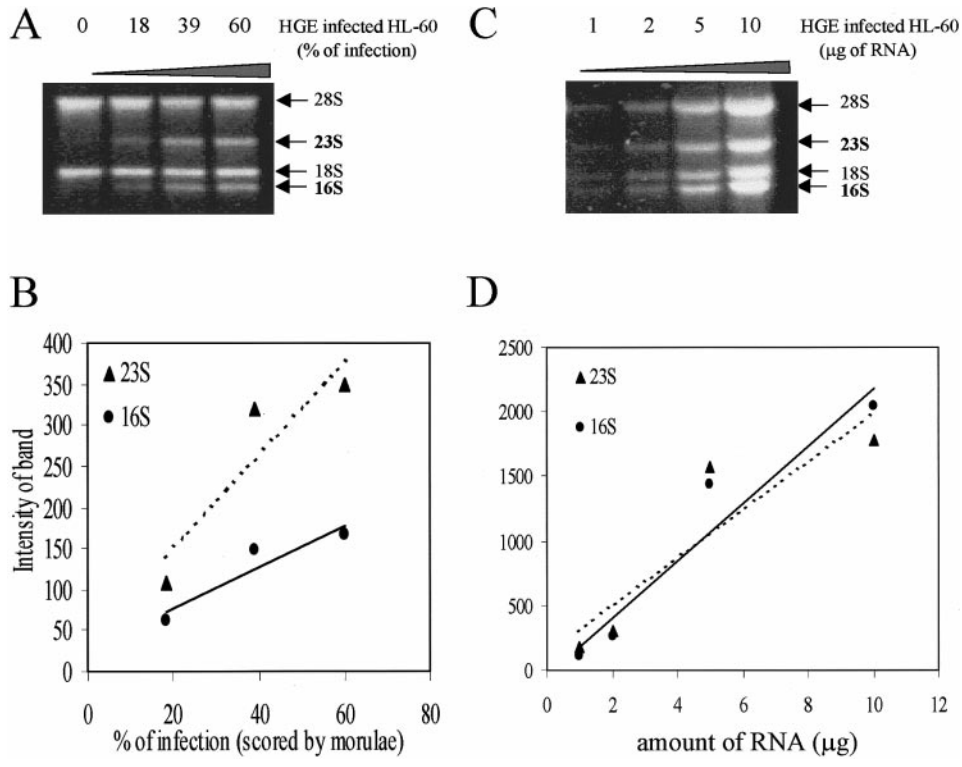


**FIG. 4.** Correlation of intensity of individual components of HGE-specific rRNA doublet with the increase of infection by HGE agent in HL-60 cells. (A) Intensity of HGE-specific rRNA in relation to magnitude of infection of HL-60 cells by the HGE agent. Lane 1, uninfected HL-60 cells; lanes 2–4, HL-60 cells infected to varying degrees with HGE isolate NY 7030. (B) A re-plot of the data in A, showing high correlation between the magnitude of infection of HL-60 cells by the HGE agent, scored on the basis of morulae, and integrated intensities of HGE agent 16S and 23S rRNA. (C and D) Correlation between different amounts of RNA applied per lane (C) and their corresponding integrated intensities (D). The RNA was isolated from 60% HGE 7030 infected HL-60 cells.

genetic information. Contrary to mRNAs that are characterized by their rarity, heterogeneity, flexibility and versatility, rRNAs are distinct in their abundance, homogeneity, stability and high levels of conservation across species (30). Ribosomes and rRNA are the only ubiquitous supramolecular complexes and organelles that occur in all organisms. Analysis of rRNA gene nucleotide sequences has allowed inference to be made of the evolutionary relationship among primary lineages of life (31, 32). Differences in size, and in the number of proteins and RNA molecules have been used to characterize ribosomes from different biological systems (33).

Since the sizes of prokaryotic rRNA (16S/23S) differ significantly from their eukaryotic counterparts (18S/28S), we reasoned that such a molecular feature could serve as a basis for identifying the presence of the HGE agent in infected HL-60 cells. Accordingly, total RNA was isolated from control and HGE agent-infected HL-60 cells using a commercially available kit and procedures provided by the manufacturer. For comparison, RNA was also isolated from JM109 or HB101 bacteria. To optimize reproducibility and recovery of RNA, a number of control experiments were performed. These included (i) varying the amount of lysozyme used for bacteria lysis; a 3-fold increase in lysozyme added did not affect recovery of RNA; (ii) mixing varying quantities of JM109 with fixed number of HL-60 cells and vice versa, to ensure that extraction and recovery of bacterial and host RNA is not adversely affected by the presence of the other cell type; (iii) comparing recovery of RNA using freshly isolated versus frozen cells. The different electrophoretic pattern of RNA isolated from HL-60 cells and from cultured bacteria on agarose gels is displayed in Fig. 3A. Figure 3B shows appearance of four distinctly separated ethidium bromide stained bands corresponding to the HGE-specific and the host 18S/28S rRNAs. Of interest is that this doublet (lane 3) appears to be smaller in size than the corresponding 16S/23S rRNA doublet isolated from *E. coli* (lane 4). Figure 4 compares results of rRNA isolated from HL-60 cells infected to varying degrees with the HGE agent. The data show that increases in infection by the HGE agent are matched by corresponding increases in intensity of the ethidium bromide stained bands for the HGE-specific rRNA doublet (Figs. 4A and 4B). In cases of high infection (>50%), increases in intensity of the ethidium bromide

stained bands corresponding to the HGE-specific rRNA doublet are directly proportional to the amount of RNA loaded per lane, with as little as 1 $\mu$g total RNA clearly showing the presence of the HGE-specific rRNA doublet (Figs. 4C and 4D). In 5–10% HGE agent infected HL-60 cells, 5–10 $\mu$g of total RNA must be loaded and analyzed to clearly visualize the HGE-specific rRNA doublet (data not shown). Figures 4B and 4D show that the integrated intensity (IU) of individual components of the doublet are significantly correlated with the increase in infection, as scored by the presence of morulae (Fig. 4B), or with the amount of RNA applied per lane (Fig. 4D). Multiple analyses with both New York isolates 7030 and 7034 validate the general utility of this assay.

Several models were considered for the relationship between IU of rRNA and the HGE bacterial load. Figure 5 shows that when the respective IU of bacterial rRNAs is presented as a ratio of the summation of IUs corresponding to bacterial and host rRNAs, the ratio is significantly correlated with both the number of *E. coli* (by the equation Y = 3.0916X + 44.588, r = 0.89) and with the % of infection by the HGE agent (by the equation Y = 0.6648X + 11.764, r = 0.94). Combining these two equations permits the calculation of *E. coli* equivalents for infection by the HGE agent, using the following equation: Y (*E. coli* in $10^8$ units) = −2.573 + 0.11X (% of infection by the HGE agent).

Compared to assays currently in use to monitor the HGE agent, the method described in this communiaction offers several advantages. First, it is an extremely rapid method, typically requiring 30 min for RNA processing and an additional 90 min for RNA separation and analysis, using inexpensive and common instruments and commercially available kits. Also, the method could be easily adapted for rapid screening and identification of bacterial infection in general, and may be particularly useful in cases where culture of the bacterium proves difficult or impossible. The potential significance of this methodological improvement is that according to current estimation, most microbes (>99%) present in biological samples collected from diverse environments are not cultivated by standard methods (34–36). Second, although DNA-based PCR methods are equally rapid and sensitive, positive results may simply reflect the presence of primer-hybridizable and amplifiable nucleic acid pieces and not necessarily intact DNA derived from actively infecting pathogenic agents. By contrast, isolation and analysis of rRNA, as outlined, demonstrates phenotypic expression of HGE rRNA genes, thereby obviating the need for PCR amplification. This in turn implies that at the time when the host cells are harvested, there is likely to be active infection of the host cells by the HGE agent. Such an interpretation is supported by the observation that the relative proportion of HGE-specific rRNA doublet increases in proportion to the percentage of infection of HL-60 cells by the pathogen (Fig. 3). Also consistent with such a conclusion is previous studies showing that death of bacteria such as *E. coli* is accompanied by a rapid degradation of ribosomes (37). An added advantage of the method presented is that the HGE-specific rRNA doublets are easily visualized by staining with ethidium bromide, raising the possibility that these bands could be recovered from the excised gel pieces and subjected to rRNA fingerprinting analysis. Fine tuning of these assays conceivably could be used to track spread of infection by the HGE agent as well generation of its phenotypic divergence *in vitro*, and for isotyping of HGE isolates derived from different localities.

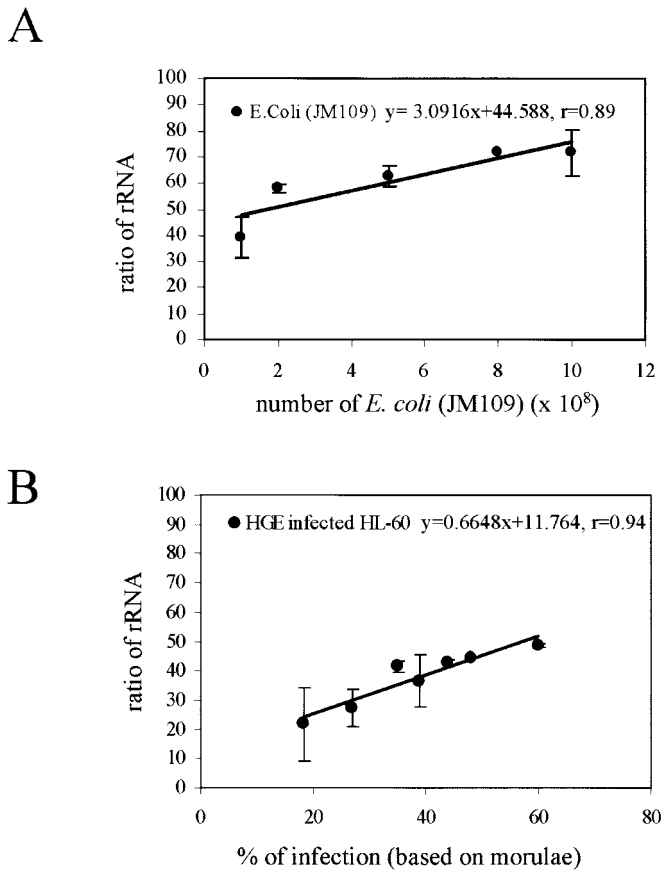


**FIG. 5.** Correlation between ratio of rRNA with number of *E. coli* and with % infection by HGE isolate 7030. Respective integrated intensity units (IU) of JM 109 rRNAs (A) and HGE agent-specific rRNA (B) presented as a ratio of the summation of IUs corresponding to bacterial and host rRNAs. The rRNA ratio is significantly correlated with both the number of *E. coli* (by the equation Y = 3.0916X + 44.588, r = 0.89) and with the % of infection by the HGE agent (by the equation Y = 0.6648X + 11.764, r = 0.94). The bars represent standard deviations of results derived from 2–4 experiments.

In conclusion, rapid laboratory confirmation of infection by the HGE agent can help ensure the most appropriate patient care. The method described in this

communication is an attractive alternative to culture and serological methods for surveillance of HGE in hospitals with heavy workloads since it offers shorter turnaround time, cost-effectiveness, and decreased risk of false positive results because of elimination of need for repeated amplifications. The assay also allows for the reliable quantification of HGE bacterial burden, expressed as equivalents of *E. coli*, and is not limited by availability of antisera stocks. When combined with analysis of host rRNA, the data obtained could be standardized in order to compare the bacterial burdens of different clinical samples. Since the method relies on *E. coli* as the reference for standardization, it is easily disseminated to other laboratories.

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