Cell Extract-Containing Medium for Culture of Intracellular Fastidious Bacteria

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The culture of fastidious microorganisms is a critical step in infectious disease studies. As a proof-of-concept experiment, we evaluated an empirical medium containing eukaryotic cell extracts for its ability to support the growth of *Coxiella burnetii*. Here, we demonstrate the exponential growth of several bacterial strains, including the *C. burnetii* Nine Mile phase I and phase II strains, and *C. burnetii* isolates from humans and animals. Low-oxygen-tension conditions and the presence of small hydrophilic molecules and short peptides were critical for facilitating growth. Moreover, bacterial antigenicity was conserved, revealing the potential for this culture medium to be used in diagnostic tests and in the elaboration of vaccines against *C. burnetii*. We were also able to grow the majority of previously tested intracellular and fastidious bacterial species, including *Tropheryma whippelii*, *Mycobacterium bovis*, *Leptospira* spp., *Borrelia* spp., and most putative bioterrorism agents. However, we were unable to culture *Rickettsia africae* and *Legionella* spp. in this medium. The versatility of this medium should encourage its use as a replacement for the cell-based culture systems currently used for growing several facultative and putative intracellular bacterial species.

Microbial culture constitutes the foundation of modern infectious disease study methodology. The isolation and culture of a disease-causing agent are a keystone of Koch's postulates, and the isolation of microorganisms in pure culture is crucial for diagnosis in medical microbiology. Microbial culture facilitates the development of diagnostic tools and allows for characterization of the genome, antibiotic susceptibility, and pathogenicity of the microorganism. At the end of the 19th century, the first culture media were developed empirically using extracts from yeast, juice, and various animal extract filtrates. These media enabled the extensive microbiological characterization of bacteria (1). At the beginning of the 20th century, following the protocol for the culture of viruses in animals, embryonated eggs, and cells, intracellular bacteria, such as *Rickettsia* species and *Coxiella burnetii*, were isolated and cultured (1). However, the inability to scale up the culture of these bacteria has dampened subsequent efforts to study them. Therefore, the establishment of axenic culture methods for these pathogens would have a significant impact on the medical and biological research communities that are working on these diseases. At the end of the 20th century, novel strategies for the culture of intracellular bacteria were proposed. *Tropheryma whippelii*, an intracellular bacterium causing Whipple's disease, was grown in culture medium supplemented with amino acids. This medium was designed after sequencing of the *T. whippelii* genome, which lacks genes encoding the enzymes involved in the synthesis of some amino acids (2). Recently, *C. burnetii*, the intracellular causative agent of Q fever, has been described as being cultivatable in an acellular chemically defined medium (3, 4). This medium was designed after a comprehensive systematic analysis was conducted of the nutrient consumption of *C. burnetii* under various oxygen tension conditions, which identified the bacterium as being microaerophilic (3, 4). The *in vitro* culture of "*Candidatus Pelagibacter ubique*," a very common seawater bacterium, was found to be particularly challenging, and an empirically formulated medium containing seawater has allowed for the *in vitro* cultivation of this bacterium (5). An empirically designed diffusion chamber method has allowed the culture of many bacterial

species previously thought to be uncultivable (6). These chambers consist of an extracted block of sand covered with seawater and are delimited by permeable membranes that allow nutrients and other environmental chemicals to enter the chamber while preventing the bacteria from escaping (6).

Culturomics, a strategy for enhancing cell culture techniques using broad-spectrum media and rapid matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry, is an emerging field that requires the formulation of new culture media (7). Here, we describe a eukaryotic cell extract-based universal empirical medium for the culture of the most intracellular and fastidious bacterial species, including *C. burnetii*, *T. whippelii*, *Mycobacterium bovis*, *Leptospira* spp., *Borrelia* spp., and most putative bacterial bioterrorism agents. This empirical medium was obtained through proof-of-concept formulation and resulted in the culture of *C. burnetii* under mesophilic conditions.

MATERIALS AND METHODS

Cultivation and purification of *C. burnetii* strains. The principal *C. burnetii* strains used in this study were the virulent Nine Mile phase I strain and the avirulent Nine Mile phase II strain, both designated RSA493 (8). Other *C. burnetii* strains included the phase II Q212, Henzerling, Priscilla, Dyer, scurry Q217, and CB92 strains (9) (see Table S1 in the supplemental material). All *C. burnetii* strains were cultured in confluent Vero cells (ATCC-CCL-81) (African green monkey kidney cells) grown in minimum essential medium (MEM) (Life Technologies, Saint-Aubin, France) supplemented with 4% fetal bovine serum (FBS) (Life Technologies,

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Saint-Aubin, France) and 1% L-glutamine (Life Technologies, Saint-Aubin, France) at 37°C in the presence of 5% CO₂. Bacteria from infected Vero cells were harvested by scraping with glass beads and centrifuging at 7,500 rpm at 4°C for 10 min (Heraeus Multifuge ×1R and Fiberlite F15-8×50cy rotor; Thermo Scientific, Saint-Herblain, France). After adding 10 ml of phosphate-buffered saline (PBS), the pellet was sonicated (Qsonica Q700 sonicator; Delta Labo, Avignon, France) three times for 1 min each time. The sample was then centrifuged at 1,000 rpm at 4°C for 10 min to remove cell debris. The supernatant was filtered using a membrane with 0.8-μm pores (Millipore, Molsheim, France). The filtrate was centrifuged at 7,500 rpm at 4°C for 10 min and the pellet was washed three times with PBS before inoculation. The amount of inoculum used was normalized by counting bacteria using Gimenez staining. A conversion factor was established based on the number of bacteria observed per field by light microscopy at ×100 magnification. The concentration of bacteria was determined using the assumption that one bacterium observed per field by microscopy at ×100 reflects a population of 10⁴ bacteria/ml (10).

Maintenance of Vero cell cultures. Vero cells are derived from the kidneys of African green monkeys (*Cercopithecus aethiops*) (11). Vero cells were propagated in MEM supplemented with 4% FBS and 1% L-glutamine at 37°C in the presence of 5% CO₂. It is extremely important to monitor Vero cells and to subculture them because they stop growing and start to senesce when they form a confluent monolayer. Depending on the number of cells seeded and the flask size, actively growing Vero cell cultures double approximately every 24 h (11). A confluent monolayer of Vero cells in 150-cm³ flasks is usually obtained within 3 days. Prior to collecting the cells for Vero cell extract-based medium (VCEM) preparation, the quality of the confluent monolayer was checked by optical microscopy.

Preparation of liquid VCEM. Vero cells cultured in M4 medium to subconfluency in 150-cm³ flasks (approximately 3.7×10^8 cells) as described above were detached from the flask by rocking the flask in the presence of sterile glass beads, followed by sonication of the cells (3 times for 1 min each time with 8,500 to 12,000 J delivered to the sample) collected in resuspension medium (M4). To ensure efficient sonication, 1 ml of the obtained VCEM was spread on the confluent shell vial (12), and VCEM was confirmed when no cellular growth was observed. The cells were then centrifuged three times at a slow speed (1,000 rpm for 10 min at 4°C). The supernatant containing the cell extract was sterilized by filtering through a 0.22-μm membrane filter and was directly used for experiments or lyophilized for long-term storage (Leybold Heraeus, Lyovac GT2 freeze dryer lyophilizer, Villebon-sur-Yvette, France). In total, one flask of 150 cm³ produced approximately 30 ml of VCEM with pH 6.8 to 7.2. We checked for Vero cell confluence and for possible contamination. Most often, VCEM was freshly prepared before use and directly used for bacterial inoculations. In some cases, VCEM had been stored in the refrigerator for a maximum of 1 week or lyophilized as described previously (13).

Inoculation of *C. burnetii* and the characterization of growth in VCEM. *C. burnetii* was quantified as previously described (10). A total of 10⁴ to 10⁶ bacteria/ml were placed into a 25-cm³ cell culture flask containing 9 ml of liquid VCEM and were incubated under microaerophilic conditions (CampyGen Compact; Oxoid, Basingstoke, Hampshire, United Kingdom) at 37°C in the presence of 5% CO₂. Every 48 h, the infected VCEM was transferred into a 15-ml Falcon tube and centrifuged (7,500 rpm for 10 min at 4°C), and 9 ml of fresh VCEM, prepared as described above, was added to the pellet. The subcultures were obtained by splitting each infected medium and adding 5 ml of fresh medium. Sampling was performed on day 0 (D0, control), D1, D2, D3, D4, D5, D6, and D8. In some cases, the culture was maintained until D15 (for Nine Mile phase I and II strains). The growth of *C. burnetii* was quantified by counting Gimenez- or acridine orange-stained bacteria by light microscopy as described previously (14). Bacterial growth was also estimated by reverse transcriptase quantitative PCR (RT-qPCR) using primers and probes that were specific to the *rpoZ* gene of *C. burnetii*. Total bacterial RNA was extracted using the RNeasy minikit (Qiagen, Courtaboeuf, France), ac-

cording to the manufacturer's protocol. One-Step RT-PCR with Platinum *Taq* (Life Technologies, Saint-Aubin, France) was performed using a LightCycler 1.1 instrument (Roche Diagnostics, Meylan, France), according to the manufacturer's instructions. DNA standards were run in parallel to quantify *rpoZ* expression as the number of cDNA copies. It is important to note that we systematically checked for the infectivity of *C. burnetii* grown in VCEM by inoculating Vero cells. The bacteria were able to replicate in cells, and their infectious phenotype was confirmed by evaluating their serological status.

Clinical isolates of *C. burnetii*. Several different strains of *C. burnetii* were tested for growth in empirical medium. We attempted to isolate *C. burnetii* from 43 different human and animal clinical samples, including those obtained from cardiac valves, liver biopsy, bone marrow, blood, and spleen biopsy. The infected organs were treated as previously described (12) before being inoculated into VCEM in shell vials. Sampling frequency was every 2 days. *C. burnetii* growth was monitored for 30 days after incubation by Gimenez and acridine orange staining and by real-time PCR using the primers for insertion element 1111 (*IS1111*) as previously described (15).

Global characterization of the VCEM. The growth of *C. burnetii* in the cell extract-supplemented liquid medium was evaluated as described above using Vero cells, human lung fibroblasts (MRC5), murine fibroblasts (L929), and human erythroleukemia cells (HEL). To further characterize VCEM, we performed ultrafiltration using a membrane with a pore cutoff of approximately 3,000 Da (YM-3; Millipore, Molsheim, France) under constant pressure (<300 kPa). A fraction of the ultrafiltrate (F) (<3,000 Da) and the concentrated medium (C) (>3,000 Da) were inoculated with *C. burnetii* as described above.

Mass spectrometry. We used MALDI-TOF mass spectrometry to identify the *C. burnetii* that was grown in VCEM. Measurements were taken using the Microflex II (Bruker Daltonics, Wissembourg, France). One milliliter of inoculated liquid empirical medium was centrifuged at 7,500 rpm for 10 min to concentrate the bacteria. The supernatant was discarded, and the pellet was washed in 200 μl of sterile distilled water, vortexed, and then centrifuged again at 7,500 rpm for 10 min. The subsequent steps (preparation of the matrix solution and the deposits and analysis of the sample) were performed as previously described (16). For each MALDI-TOF profile, we calculated the quality score by analyzing the peaks between 900 and 3,000 Da.

Electron microscopy. The Nine Mile phase I and II strains cultured in cells and in liquid VCEM were examined by transmission electron microscopy using a Philips-Morgagni 368D electron microscope (Fei Company, Eindhoven, The Netherlands) as previously described (17). The pellet of *C. burnetii* was subjected to negative staining. Briefly, 40 μl of sample was adsorbed onto carbon-coated copper grids (for 15 min at 37°C), stained with 1% (wt/vol) aqueous uranyl acetate for a few seconds, dried on the grids, and examined by transmission electron microscopy. We measured the size of 20 randomly selected bacteria grown in VCEM and 20 randomly selected bacteria cultured in cells and purified with sucrose as previously described (18).

ELISA and indirect immunofluorescence assay. An enzyme-linked immunosorbent assay (ELISA) was used to measure the anti-phase II IgM present in the sera of *C. burnetii*-infected patients. *C. burnetii* was purified by centrifuging the bacteria grown in VCEM or by sucrose gradient purification of *C. burnetii* grown in cells as previously described (18). We tested 10 serum samples from patients with Q fever and 10 serum samples from patients without Q fever as confirmed by an immunofluorescent antibody (IFA) assay, which is the standard method for Q fever diagnosis (18). All steps of the ELISA were performed as previously described (19), with the following modifications: (i) the amount of antigen added per well was 0.5 μg, and (ii) the serum was diluted 1:100 in PBS-Tween 0.1% supplemented with 3% bovine serum albumin (BSA). The mean value of the 10 negative sera $\pm$ 2 standard deviations (SD) was set as the basal optical density cutoff value, and the results were expressed as the normalized ratio (optical density at 405 nm/basal cutoff value). The IFA assay was

TABLE 1 Delay of bacterial culture in liquid VCEM for all of the fastidious bacteria tested compared to delay of culture using conventional media^a

Bacterial species	Strain	Style of life	Delay of growth in liquid VCEM ($\times \log_{10}$) (days)	Type of conventional medium ^b	Delay of growth in conventional medium	Reference(s)
<i>Tropheryma whippelii</i>	Twist	Intracellular facultative	2	Specific axenic medium	5–6	2
<i>Bartonella henselae</i>	Houston-1 (ATCC 49882 ^T)	Intracellular facultative	3	Columbia blood agar	5–15 days	30
<i>Bartonella quintana</i>	Oklahoma strain	Intracellular facultative	No growth	Columbia blood agar	5–15 days	30
<i>Francisella tularensis</i>	Holartica LVS	Intracellular facultative	20	Columbia blood agar	2–14 days	31
<i>Rickettsia africae</i>	CSUR R15	Intracellular obligate	No growth	XTC, HEL cells	7 days	10
<i>Mycobacterium bovis</i>	BCG CIP 105050	Intracellular facultative	8	Blood agar	14–24 days	32, 33
<i>Leptospira interrogans</i>	M 20	Free-living	4	Ellinghausen-McCullough-Johnson-Harris medium	1–13 wk	34
<i>Borrelia crocidurae</i>	CSUR P235	Free-living	14	BSK-H	1–6 wk	34
<i>Borrelia burgdorferi</i>	B31	Free-living	No growth	BSK-H	1–6 wk	34
<i>Brucella melitensis</i>	B115	Free-living	2	Columbia blood agar	2–21 days	
<i>Yersinia pestis</i>	EV76	Free-living	2	Trypticase soy broth MacConkey agar	1 day	35
<i>Vibrio cholerae</i>	CSUR P238	Free-living	2	TCBS agar	1 day	36
<i>Bacillus anthracis</i>	CIP 77.2	Free-living	2	Sheep blood agar		37
<i>Shigella dysenteriae</i>	CIP 57.28	Intracellular facultative	2	BHI broth or agar	1 day	38
<i>Bordetella pertussis</i>	CSUR P237	Free-living	2	Bordet-Gengou medium	5–6 days (at 37°C)	39
<i>Campylobacter fetus</i>	CSUR P236	Free-living	5	Columbia blood agar	2–3 days (in microaerophilic atmosphere)	40, 41
<i>Campylobacter jejuni</i>	CSUR P239	Free-living	5	Columbia blood agar	2–3 days (in microaerophilic atmosphere)	40, 41
<i>Legionella pneumophila</i>	CIP 103854 ^T	Intracellular facultative	No growth	Buffered charcoal yeast extract medium	4–10 days	42
<i>Legionella drancourtii</i>	ATCC 50991	Intracellular facultative	No growth	Buffered charcoal yeast extract medium	2–4 days	43
<i>Legionella anisa</i>	ATCC 35291	Intracellular facultative	No growth	Buffered charcoal yeast extract medium	2–4 days	44
<i>Legionella micdadei</i>	ATCC 33218	Intracellular facultative	No growth	Buffered charcoal yeast extract medium	4–10 days	44

^a The threshold for significant growth was set at a 10-fold increase in the bacterial population.

^b XTC, *Xenopus* tissue culture cells; HEL, human erythroleukemia cells; BSK-H, Barbour-Stoenner-Kelly H medium; TCBS, thiosulfate-citrate-bile salts-sucrose agar; BHI, brain heart infusion.

also performed for the Nine Mile phase I and phase II antigens using a homemade rabbit polyclonal anti-serum directed against the Nine Mile phase II strain and with 2 serum samples obtained from patients with confirmed Q fever infection.

Extraction of proteins for 2D-DIGE analysis and protein identification. The *C. burnetii* Nine Mile phase II strain was grown in Vero cells (20), acidified citrate cysteine medium (ACCM) (3), and VCEM. Bacteria in the exponential phase of growth were collected by centrifugation or were isolated using sucrose gradients (Euromedex, Souffelweyersheim, France), and protein extraction was then carried out as previously described (20). Two-dimensional differential gel electrophoresis (2D-DIGE) was used to compare the protein expression profile of *C. burnetii* purified from Vero cells with that of *C. burnetii* grown in VCEM or ACCM. Four individual samples from each culture method were prepared for minimal labeling with Cy3 and Cy5 fluorescent dyes as previously described (21). The images were analyzed using the Progenesis SameSpots software version 4.1.3 from Nonlinear Dynamics (Newcastle upon Tyne, United Kingdom), as per the manufacturer's instructions. Significant differences in 2D spot abundance were identified as having an analysis of variance (ANOVA) score with a *P* value of ≤ 0.05 and a change in intensity of ≥ 1.5 -fold (see Table S2 in the supplemental material). Spots observed to be significantly different were digested and identified by mass spectrometry as previously described (21).

Growth of other bacterial species in VCEM. Similar protocols were used to test liquid VCEM for the growth of other fastidious bacteria that are routinely grown in our laboratory and some bacterial species that are classified as potential bioterrorism agents. These species tested are summarized in Table 1. The bacterial inocula were obtained by harvesting colonies, except for *Borrelia crocidurae* and *Borrelia burgdorferi* (Barbour-Stoenner-Kelly H medium; Sigma, Saint-Quentin-Fallavier, France), *Leptospira interrogans* (Ellinghausen-McCullough-Johnson-Harris modified medium; Bio-Rad Laboratories, Marnes-la-Coquette, France), and *T. whippelii* (2), which were collected by centrifugation. For *L. interrogans*, *B. crocidurae*, and *B. burgdorferi*, aliquots of 10^6 bacteria were inoculated into 9 ml of liquid medium and incubated at 32°C in the presence of 5% CO₂.

Campylobacter fetus and *Campylobacter jejuni* were incubated under microaerophilic conditions (CampyGen Compact, Oxoid, Basingstoke, Hampshire, United Kingdom) at 37°C in the presence of 5% CO₂. All other bacteria were incubated at 37°C in the presence of 5% CO₂. Every 2 days, the infected medium was centrifuged and 9 ml of fresh medium was added to the pellet as described previously. Sampling for growth was performed on D0 (control), D2, D4, D6, D8, and D10, and in some cases, samples were taken through D30. The growth of bacteria was monitored microscopically by acridine orange, Gram or Gimenez staining as described previously (14). In addition, the growth of each bacterium was quantified by qPCR when primers and probes were available (see Table S3 in the supplemental material). DNA was extracted from a 200- μ l sample of inoculated liquid medium with 500 μ l of Chelex, as recommended by the manufacturer. The qPCR was performed using a CFX instrument (Bio-Rad, Marnes-la-Coquette, France) and QuantiTect mix (Qiagen, Courtaboeuf, France). All samples were tested for purity, and each sample was diluted (1:10 and 1:100) to avoid the effect of PCR inhibitors. The threshold for significant growth was set at a 10-fold increase in the bacterial population. In parallel, tested bacteria were grown in conventional media. All experiments were performed in triplicate.

Growth of *C. burnetii* and *T. whippelii* in ACCM-2. The *C. burnetii* Nine Mile II strain was inoculated into ACCM-2, a medium designed for the axenic culture of *C. burnetii* (4) under microaerophilic conditions (CampyGen Compact, Oxoid, Basingstoke, Hampshire, United Kingdom) at 37°C in the presence of 5% CO₂.

The *T. whippelii* strains Twist, Endo5, and Slow2 were grown in a liquid axenic medium and collected 8 to 10 days postinfection as previously described (2). In parallel, the rich inocula of *T. whippelii* strains Endo5 and Slow2 (10^6 bacteria/ml) were used for 20 days of culture in the axenic medium ACCM-2 as previously described (4). Bacterial growth was observed by staining and qPCR as described above.

RESULTS

Cell extract-containing medium supports the acellular growth of *C. burnetii*. Liquid VCEM was inoculated with *C. burnetii* Nine

FIG 1 Growth of *C. burnetii* in VCEM. Growth of the Nine Mile phase I strain and the Nine Mile phase II strain in liquid VCEM with or without a replacement with fresh medium (RM) every 48 h. The growth of *C. burnetii* was monitored by microscopy using acridine orange staining.

Mile phase I and phase II strains, and viable bacteria were counted by acridine orange staining. Active bacterial replication (orange-staining bacteria) was observed 24 to 48 h postinoculation (14). However, the proportion of inactive (green-staining bacteria) *C. burnetii* gradually increased after 48 h (Fig. 1). This rapid replication of the bacterium prompted us to change the medium every 2 days to avoid reaching growth-limiting conditions (Fig. 1). *C. burnetii* that had grown exponentially within 6 days was able to persist in the medium. In addition, VCEM supported the growth of both the virulent Nine Mile I and the avirulent Nine Mile II *C. burnetii* strains. These results were confirmed with reverse transcriptase qPCR, and bacterial identity was confirmed by MALDI-TOF mass spectrometry (Fig. 2). Three subcultures were obtained from the Nine Mile phase II strain by subculturing this strain every 2 days. This experiment was performed independently by 3 different investigators. Similar results were obtained for the other *C. burnetii* strains Priscilla, Dyer, Scurry Q217, Q212, and CB92.

Isolation of *C. burnetii* on VCEM from infected clinical samples. In the initial stages of VCEM development, we had already started to work on *C. burnetii* isolation from clinical specimens that were sent to our laboratory for diagnosis. Thus, we performed in parallel the culture of bacteria from 43 human and animal samples using the standard reference protocol for *C. burnetii* isolation and experimental methods using VCEM. As demonstrated in Fig. 3, VCEM supports the isolation and culture of approximately 60% of the clinical samples. The clinical isolate *C. burnetii* 57811 was maintained for 75 days in VCEM and 10 subcultures could be obtained. The identification of *C. burnetii* by Gimenez staining or indirect immunofluorescence using acridine orange was found to vary within samples: the clustered bacteria regrouped by 10 or 20 were visible for some samples as early as 6 days postinfection and for others at 15 or 24 days postinfection. For some samples, qPCR-based quantification of the DNA copies was also found to vary

within the samples. Moreover, in some cases, although the qPCR was positive, the microscopic observation was unsuccessful, and vice versa. In addition, VCEM supports the isolation and culture of *C. burnetii* from clinical specimens.

Global characterization of empirical medium. To determine the role of the cell extract in VCEM, we first compared *C. burnetii* growth in the complete system and in MEM supplemented with 4% FBS and 1% L-glutamine. No bacterial growth was observed in M4 medium alone. Next, we tested different preparations of empirical media made from different cell lines. Several eukaryotic cell line extracts, including Vero, MRC5, and L929, supported the growth of the *C. burnetii* Nine Mile phase II strain. However, the HEL cell extract was less amenable to *C. burnetii* growth for reasons that are not yet known. These results suggest that the cell extract is critical for *C. burnetii* growth. However, its composition is very complex, and sophisticated tools and further studies are required to elucidate the exact role of the cell extract in bacterial growth. To decipher in detail the composition of the empirical medium, we used ultrafiltration to separate the medium into two fractions: the ultrafiltrate fraction, mainly containing small hydrophilic molecules and short peptides (molecules < 3 kDa), and the concentrate fraction containing the essential proteins from FBS (e.g., albumin) (molecules > 3 kDa). The bacterium was able to grow in the ultrafiltrate, suggesting that proteins larger than 3 kDa that are present in FBS are not essential for bacterial growth. Ultrafiltration of the VCEM revealed that small hydrophilic molecules and short peptides (<3 kDa) are critical for bacterial growth, as was previously described for *Psychrobacter* spp. (22). These small molecules and peptides remain to be identified.

The cell-derived medium deteriorates upon long-term storage. However, it is possible to preserve freshly prepared medium in the refrigerator for up to 1 week. When healthy cells and highly purified reagents were used, we did not observe significant differences

FIG 2 Identification of *C. burnetii* by mass spectrometry. MALDI-TOF spectra of the Nine Mile phase II strain obtained from liquid VCEM culture (a) and ACCM culture (b) and purified from Vero cells (c).

between assays that were conducted with freshly prepared versus 1-week-old medium. However, the quality of cell-derived medium varies between different batches of cell culture.

We carried out several assays to establish the best way of preserving the cell-derived medium. Lyophilization was found to be

the preferred method because it also allows for the standardization of medium preparation for use in diagnostic assays.

Characterization of *C. burnetii* grown in VCEM. We characterized *C. burnetii* grown in VCEM by electron microscopy and did not observe any morphological differences from *C. burnetii* grown in Vero cells. The average length-to-width ratio for 20 randomly selected bacteria was 2.05 for the Nine Mile phase I strain and 2.17 for the Nine Mile phase II strain when grown in VCEM, compared to 2.34 for the Nine Mile phase I strain and 1.93 for the Nine Mile phase II strain cultured in Vero cells (see Fig. S1 in the supplemental material). *C. burnetii* grown in VCEM was also analyzed by MALDI-TOF (23), which showed that the spectra of *C. burnetii* Nine Mile phase II were similar to those obtained for *C. burnetii* grown in Vero cells and *C. burnetii* grown in ACCM (Fig. 2). The antigenicity of the Nine Mile phase II strain grown in our medium or in Vero cells (18) was tested by ELISA and by IFA assays using human serum identified as positive or negative for *C. burnetii* infection. The ELISA results showed similar specificities for the Nine Mile phase I and phase II antigens and showed better sensitivity for antigens obtained from *C. burnetii* cultured in VCEM than for those cultured in Vero cells ($P = 0.003$ using

FIG 3 Isolation of *C. burnetii* from a human cardiac valve specimen. *C. burnetii* was successfully cultured from a human cardiac valve and isolated at 17 days postinoculation in VCEM. *C. burnetii* appears regrouped in clusters of 10 to 20 viable orange bacilli with acridine orange staining ($\times 100$).

FIG 4 *C. burnetii* serological assays. (a) ELISA with positive and negative sera from patients using the *C. burnetii* antigen purified from *C. burnetii* grown in cell culture systems and antigens from *C. burnetii* grown in VCEM. The results are expressed as a normalized ratio (NR) (see Materials and Methods for explanation). An NR of <1 was assigned for negative sera (green), and an NR of >1 was assigned for positive sera (red). (b) IFA assay performed with polyclonal rabbit antiserum on Nine Mile phase I (right) and phase II (left) strain antigens grown in liquid VCEM (bottom) and purified from Vero cells (top).

Student's *t* test (Fig. 4a). The reactivities of specific rabbit polyclonal antibodies and patient sera were similar for both Nine Mile phase I and phase II antigens when tested by IFA assay (Fig. 4b). These results suggest that the preservation of the antigenicity of bacteria is potentially useful for diagnostic tests and for rapid and effective vaccine development (24, 25).

The protein expression profiles at the exponential phase of growth in *C. burnetii* grown in Vero cells, ACCM, and VCEM were compared by 2D-DIGE (Fig. 5). Thirty spots were upregulated and 11 spots were downregulated when bacteria were grown in our medium compared to culture in Vero cells. In contrast, 36 spots were downregulated and 4 upregulated when bacteria were grown in ACCM (3) compared to Vero cells (Fig. 5; see also Table S2 in the supplemental material). The differentially expressed proteins were primarily involved in oxidative stress regulation. This was an expected result because of the preference of *C. burnetii* for microaerophilic conditions. *C. burnetii* upregulates the expression of antioxidant proteins under oxidative stress, which can vary based on the oxygen tension conditions within the culture system. Because the oxygen tensions in VCEM and the Vero cell culture system are similar, the expression levels of antioxidant proteins by *C. burnetii* were comparable in these two systems. In contrast, because ACCM has a low pH and low oxygen tension (2.5% versus 7.5 to 10% partial O_2 pressure [pO_2] in our medium), the oxidative stress protein expression was downregulated. This result is consistent with the results of the transcriptomic analysis (3, 26).

Growth of *C. burnetii* in ACCM-2. The growth of the *C. burnetii* Nine Mile phase II strain in ACCM-2 (4) was comparable to that in VCEM as quantified by acridine orange staining within 6 days (see Fig. S2 in the supplemental material). We were able to maintain *C. burnetii* cultures in VCEM and ACCM-2 for 60 days.

Other bacteria. We tested liquid VCEM for its ability to support the growth of other fastidious bacteria and putative bacterial bioterrorism agents (listed at www.bt.cdc.gov/bioterrorism). We considered a 10-fold increase in bacterial growth as evaluated by microscopy and by qPCR to be significant (Table 1; see also Table

S1 in the supplemental material). We observed rapid and significant growth at 48 h postinoculation for *Yersinia pestis*, *Vibrio cholerae*, *Bacillus anthracis*, *Shigella dysenteriae*, *Brucella melitensis*, and *Bordetella pertussis*. *Bartonella henselae* attained significant growth in 72 h, but the *Bartonella quintana* population did not grow in VCEM. Among the spirochetes, *B. crocidurae* reached our threshold for significant growth in 14 days, while *L. interrogans* grew in 4 days. However, the *B. burgdorferi* population did not grow in VCEM. *M. bovis* and *Francisella tularensis* attained significant growth in 8 and 20 days, respectively. Under microaerophilic conditions, *C. fetus* and *C. jejuni* grew in 5 days. We also observed the growth of *T. whipplei* in our medium after 2 days (see Fig. S3 in the supplemental material). In contrast to growth in our previously described medium (2), *T. whipplei* did not arrange in filamentous ropes in VCEM. Fourteen days after inoculation in VCEM, we were also able to isolate a strain of *T. whipplei* directly from the cardiac valve sample of a patient who presented with endocarditis. *Legionella pneumophila*, *Legionella drancourtii*, *Legionella anisa*, *Legionella micdadei*, and *R. africae* failed to grow in VCEM.

DISCUSSION

In this study, we have demonstrated the ability of *C. burnetii* to grow in VCEM. The ultrafiltrate fraction of this medium, which contains only molecules of <3 kDa, supports the growth of *C. burnetii*. However, prior to the identification of the essential growth factor, we found that refreshing the culture medium every 2 days slightly increased the growth rate of *C. burnetii*. Furthermore, our results show that this medium preserves the natural antigenicity of *C. burnetii* and that the virulent form of *C. burnetii* (Nine Mile phase I) also grows in it. This finding suggests that this medium can be used for the production of antigens from *C. burnetii* for use in diagnostic tests and vaccine development (24, 25). *C. burnetii* grown in VCEM retained its infective phenotype as in cells. VCEM represents an alternative to growing fastidious bacteria in chemically defined axenic medium that has been described

FIG 5 Representative 2D-DIGE analysis of *C. burnetii* proteins. (A) Each sample of *C. burnetii* grown in Vero cells and in ACCM was labeled with Cy5 and Cy3, respectively, and (B) each sample of *C. burnetii* grown in Vero cells, VCEM, and a pooled reference sample was labeled with Cy5, Cy3, and Cy2, respectively, and the samples were then separated on the same 2D-DIGE system. Three images were obtained from each gel, and an overlay of the dye-scanned images was obtained. Selected protein spots exhibiting an ANOVA score of ≤ 0.05 and a change in intensity of ≥ 1.5 -fold are indicated by arrows and numbers and are listed in Table S1 in the supplemental material.

previously (3), which was based on the landmark discovery by Hackstadt et al. (27, 28) that *C. burnetii* axenic metabolic activity is significantly enhanced in an acidic buffer (approximately pH 4.5). However, despite having a neutral pH (6.8 to 7.2), VCEM supports *C. burnetii* growth, suggesting that *C. burnetii* grown in VCEM does not require acidic activation of its metabolic path-

ways. This finding is also a significant difference between VCEM and ACCM (3), the latter of which is highly specialized for *C. burnetii* and does not support the growth of *T. whipplei*. Preliminary results showed that *C. burnetii* grows slightly better in ACCM-2 (4) than in VCEM and that the improvement of ACCM allowed the growth of approximately 10 bacteria. However, the

early work of Omsland et al. (3) has supported the use of *C. burnetii* inoculum in the range of 10^4 to 10^6 bacteria/ml, which is comparable with our experimental conditions. We were successful in the isolation of *C. burnetii* strains from clinical samples. However, we believe that the encouraging results of using VCEM can be improved further. Therefore, it is necessary to identify the specific factors necessary for the growth of *C. burnetii*. Several attempts to decipher the composition of VCEM using nano-liquid chromatography coupled to tandem mass spectrometry (nano-LC-MS/MS) have failed due to the noisy background resulting from M4 masking the factors present in the cell extract. In addition, a successful characterization of the ultrafiltrate fraction remains to be carried out.

Our medium also supports the growth of *T. whipplei* and several other facultative intracellular bacteria. To the best of our knowledge, this is the first medium that permits the growth of two spirochetes belonging to different phyla.

The main advantage of VCEM over other esoteric media is its ability to recover the growth of several intracellular and fastidious bacteria from clinical samples in a pattern similar to that observed during disease. Therefore, it can be used as a “clinical kit.” We have successfully isolated *C. burnetii* from diverse clinical specimens and *T. whipplei* from an infected cardiac valve specimen. When comparing the growth of fastidious bacteria in VCEM and conventional medium (Table 1), the results are variable. In some cases, the culture duration was shortened (e.g., *T. whipplei* replicated within 2 days in VCEM but required 5 days in Dulbecco’s modified Eagle’s medium [DMEM]/Ham’s F-12), while in other cases, it was longer (*Y. pestis* needed 2 days in VCEM and only 1 day in Trypticase soy broth). However, the main utility of VCEM is the success with the primary attempt for clinical sample isolation. The success of VCEM with clinical applications also justifies its elaborate preparation protocol, including Vero cell cultures and maintenance, and the standardization of large-scale VCEM production. The preservation of lyophilized VCEM has been shown to be efficient, and *C. burnetii* was demonstrated to grow in regenerated VCEM, although slightly less efficiently than in the freshly prepared medium. Thus, the versatility of this medium supports its use in the clinic as a replacement for cellular culture systems for the growth of several facultative and strictly intracellular bacteria, including most of the putative bioterrorist agents.

Another important advantage is the potential to optimize VCEM for culturing other intracellular fastidious bacterial species by testing variations in temperature, the atmosphere of incubation, supplementation with nutrients, and growth factors required by the bacteria mentioned in this study. In addition, VCEM may be considered a basic formulation for future development.

Actually, bacterial culture techniques are in a process of a rebirth. The popular idea that metagenomics will render bacterial culture methods obsolete is losing steam. Bacterial culture is essential for the study of bacterial genomes (29), pathogenicity, and antibiotic susceptibility, and for the design of more advanced diagnostic tools. A major limitation of the study of fastidious intracellular bacteria has been the lack of eukaryotic cells supporting their growth on multiple esoteric media. Our empirical medium is easy to use and versatile, allowing for many applications for isolation and culture of unknown fastidious bacteria from clinical samples.

In conclusion, we present an empirically designed broad-spectrum bacterial culture medium containing eukaryotic cellular ex-

tracts that allows for the culture of numerous intracellular bacterial species. One limitation of our current medium is its inability to sustain the growth of *Rickettsia* and *Legionella* species, but further improvements in our technique will increase the number of bacterial species that can be supported by our medium.

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A patent on the medium is pending.

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D.R. conceived and designed the research, S.S., M.K., S.E., C.E., C.P., S.A., and P.W. performed the research, M.K., S.E., S.A., and D.R. analyzed the data, and M.K., S.E., S.A., and D.R. wrote the paper.

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