Title:

Bacterial sequences detected in 99 out of 99 serum samples from Ebola patients

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Evolution and clinical manifestations of Ebola virus (EBOV) infection overlap with the pathologic processes that occur in sepsis[1]. Some viruses certainly compromise the immune system, leading to a breach in the integrity of the mucosal epithelial barrier, thus allowing bacterial translocation[2, 3]. Guided by these facts, we wondered if bacteria could be involved in the pathogenesis of some of the septic shock-like symptoms typical of EBOV infected patients, something that could have a dramatic impact on the design of new treatment approaches. We decided to search for bacteria in available EBOV patient sequence datasets. Given that EBOV is an RNA virus and that, hence, some NGS sequencing experiments carried out to sequence the EBOV genomes were RNA-Seq experiments, we thought that, if there were any bacteria in patient serum, at least some bacterial RNA might probably be detected in the sequenced material from Ebola patients. Thus, we searched for bacteria in a RNA-Seq public dataset from 99 Ebola samples from the last outbreak[4], and surprisingly, in spite of the certainly suboptimal experimental conditions for bacterial RNA sequencing, we found bacteria in all of the 99 samples.

RNA-Seq dataset from Ebola patients

The datasets with the illumina reads of the samples from the Ebola outbreak published[4] were retrieved from the SRA. For each sample listed in the Supplementary Table S2 of the article[4] the Experiment sample (SRS) information and links to its SRA experiments (SRX) and runs (SRR) were retrieved using the NCBI Batch Entrez platform (http://www.ncbi.nlm.nih.gov/sites/batchentrez).

We analyzed a total of **166.710.398** reads from this 99 RNA-Seq dataset of the sequences obtained from serum from Ebola patients. Table 1 shows the number of reads analyzed and

the reads assigned to bacteria in each sample. SRA IDs corresponding to the 99 Datasets[4], description of Bioinformatics methods and additional data about statistics and frequency distribution of detected bacteria are available as Supplementary Material. We searched for the presence of bacterial 16S rRNA sequences. The reads for each sample were used as query sequences for BLASTn against a custom 16S database using MG7 metagenomics profiling tool[5]. The database was built with the GenBank sequences corresponding to the sequences published in the RDP database (https://rdp.cme.msu.edu/index.jsp). Based on the BLASTn results we performed a subsequent taxonomic assignment to the NCBI taxonomy using 2 different assignment paradigms: weighted Lowest Common Ancestor (LCA) and Best BLAST Hit (BBH). This analysis was done using MG7 metagenomics profiling tool[5]; this cloud-based bacterial community profiling tool (open source code available at GitHub https://github.com/ohnosequences/mg7) maps the input sequences against a database of bacterial 16S sequences using BLAST, and uses these hits to identify their taxonomic node through a BBH or a weighted LCA paradigm.

Given that we searched only for bacterial 16S sequences it is important to consider that the number of actual sequences from bacteria could be considerably higher since sequences from other bacterial genes would also be expressed in the samples. Figure 1 shows the percentage of the top 30 most abundant bacteria detected in the 99 samples using BBH taxonomic assignment paradigm. There are different bacterial diversity profiles both among samples and patients, although in some cases intra-patient commonalities are recognized. The top 10 most abundant taxa in all the samples (ranked using the average percentage of BBH assigned reads in all the samples) were *uncultured Streptococcus sp.*, *uncultured gamma proteobacterium*, *uncultured Pseudomonas sp.*, *uncultured actinobacterium*, *uncultured cyanobacterium*, *uncultured Firmicutes bacterium*, *uncultured Staphylococcus sp.*, *uncultured alpha proteobacterium*, *uncultured beta proteobacterium* and *uncultured Bacilli bacterium*.

Strikingly all these taxa are uncultured bacteria. Symptoms of EVD often mimic those due to bacterial sepsis (http://www.who.int/csr/disease/ebola/clinical-care-summary.pdf). Negative bacterial blood culture in EBV patients with symptoms of bacteraemia or sepsis could be related with the fact that bacteria migth be unculturable. Some samples showed frequency peaks of taxa which were not abundant in the other samples. *Uncultured Streptococcus, uncultured Staphylococcus, uncultured Actinobacterium, uncultured Clostridium or uncultured Bacilli bacterium* were some of these sample-specific peaks.

Figure 2 shows the percentage of the top 30 most abundant bacteria detected using LCA taxonomic assignment paradigm.

Tables and charts with the complete data about the bacteria detected using LCA and BBH paradigms are available as Supplementary material.

Patient evolution and bacterial load

Tables 2 and 3 and Figures 3 and 4 show EBOV **copies/ml** and **% bacterial reads** in two patients with different outcome. We selected these two patients with 4 available RNA-Seq samples taken at different points of the Ebola disease evolution. Curiously the last point of the discharged patient (G3769-4) shows a very high level of viral EBOV load and very low bacterial load. On the contrary, in the last point of the patient with fatal outcome the bacterial load was higher than the viral EBOV load.

It has been reported [6] how a patient course was complicated with Gram negative sepsis when the viral load in blood was declining. After treatment with intensive fluid resuscitation, broad-spectrum antimicrobial therapy, and ventilatory support, the patient survived. This finding is coherent with the role that we propose for bacteraemia in EVD.

New perspective in Ebola disease clinical intervention

This work could open a new perspective in both the pathogenesis and the treatment of EBOV infections. These findings suggest that sepsis therapies, specific antibiotics and mucosal epithelial barrier protectors could be considered. It is possible that, as in other immunocompromised patients, the crucial interventions would be targeting those bacteria. This is especially important in the case of EVD because there is not a specific treatment for Ebola virus.

References

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Acknowledgments

Table 1. Statistics of bacterial assigned reads

Sample ID	Analyzed reads (after filtering)	No Hit (against 16S bacterial RNA DB)	Not assigned	bacterial assigned reads (using a 16S bacterial RNA database)	% bacterial reads	outcome
EM104	927027	912988	6610	7429	0.80	Died
EM110	4892870	4891785	459	543	0.01	Died
EM111	1464419	1450936	6025	7458	0.51	Died
EM112	3648071	3644895	1335	1841	0.05	Died
EM113	964090	949001	8350	6739	0.70	Died
EM115	1222962	1206106	7259	9597	0.78	Died
EM119	2279332	2270073	4251	5008	0.22	Died
EM120	2160593	2145698	7130	7765	0.36	Died
EM121	3069339	3065083	2204	2052	0.07	Died
EM124-1	2120862	2113021	4209	3632	0.17	Died
EM124-2	755247	738574	5950	10723	1.42	Died
EM124-3	768610	762537	3194	2879	0.37	Died
EM124-4	222370	220913	591	866	0.39	Died
G3676-1	2161216	2151979	3755	5482	0.25	Died
G3676-2	2190925	2186248	1280	3397	0.16	Died
G3677-1	1577917	1577083	261	573	0.04	Died
G3677-2	1834471	1833450	217	804	0.04	Died
G3707	1545610	1513805	11116	20689	1.34	Died
G3713-2	3256482	3253707	826	1949	0.06	Died
G3713-3	2760034	2751089	3495	5450	0.20	Died
G3713-4	2936648	2932385	1588	2675	0.09	Died
G3724	3401326	3401088	116	122	0.003	Died
G3735-1	3652776	3650681	512	1583	0.04	Died
G3735-2	3900255	3899104	314	837	0.02	Died
G3752	755678	754752	255	671	0.09	Died
G3764	3566180	3565650	160	370	0.01	Died
G3770-1	2527295	2517367	2769	7159	0.28	Died
G3770-2	5208479	5199893	3032	5554	0.11	Died
G3787	681519	667834	5420	8265	1.21	Died
G3795	1099027	1089879	4185	4963	0.45	Died
G3798	821307	810289	5054	5964	0.73	Died
G3800	1974089	1946261	11317	16511	0.84	Died
G3807	1599122	1569590	13024	16508	1.03	Died
G3808	2060388	1979793	31807	48788	2.37	Died
G3814	835800	812366	10074	13360	1.60	Died
G3816	809251	770952	16939	21360	2.64	Died
G3818	2044579	2023665	7953	12961	0.63	Died
G3820	1897113	1876463	7434	13216	0.70	Died
G3822	2542423	2535011	2886	4526	0.18	Died
G3823	3418212	3409133	3089	5990	0.18	Died

G3825-1	2525303	2495191	11298	18814	0.75	Died
G3825-2	2760725	2755436	2042	3247	0.12	Died
G3826	946841	934807	4982	7052	0.74	Died
G3827	786789	776089	4211	6489	0.82	Died
G3829	1434654	1397643	12567	24444	1.70	Died
G3831	2101611	2091908	3266	6437	0.31	Died
G3834	2015904	1994714	11613	9577	0.48	Died
G3838	1564305	1556398	3078	4829	0.31	Died
G3840	2382513	2377172	2774	2567	0.11	Died
G3845	2712208	2705089	2928	4191	0.15	Died
G3846	2307278	2301597	2204	3477	0.15	Died
G3848	2489357	2484112	1949	3296	0.13	Died
G3851	1917446	1897411	10276	9759	0.51	Died
G3856-1	2189510	2188838	231	441	0.02	Died
G3856-3	1176332	1159797	9682	6853	0.58	Died
EM106	785650	774050	6352	5248	0.67	Discharged
G3670-1	1603798	1597937	2049	3812	0.24	Discharged
G3765-2	485083	478798	2320	3965	0.82	Discharged
G3769-1	1219532	1208037	4914	6581	0.54	Discharged
G3769-2	806309	798797	2696	4816	0.60	Discharged
G3769-3	1262784	1238388	11790	12606	1.00	Discharged
G3769-4	257875	257578	206	91	0.04	Discharged
G3789-1	582942	570205	5300	7437	1.28	Discharged
G3796	2093457	2081103	4615	7739	0.37	Discharged
G3799	1415467	1386254	10825	18388	1.30	Discharged
G3805-1	1231640	1193536	18366	19738	1.60	Discharged
G3805-2	293178	289975	1334	1869	0.64	Discharged
G3809	695337	672263	10351	12723	1.83	Discharged
G3810-1	1007240	979654	12563	15023	1.49	Discharged
G3810-2	769238	763558	2707	2973	0.39	Discharged
G3817	1094044	1078801	6364	8879	0.81	Discharged
G3819	764579	748395	6304	9880	1.29	Discharged
G3821	1128371	1103076	10264	15031	1.33	Discharged
G3850	480504	447848	14395	18261	3.80	Discharged
G3857	507320	499773	3090	4457	0.88	Discharged
NM042-1	755816	747548	3241	5027	0.67	Discharged
NM042-2	1257035	1226132	14582	16321	1.30	Discharged
NM042-3	439674	435580	1310	2784	0.63	Discharged
EM095	3731730	3626319	36020	69391	1.86	N/A
EM095B	2029600	2024144	2288	3168	0.16	N/A
EM096	562860	557354	1789	3717	0.66	N/A
EM098	446412	440438	1895	4079	0.91	N/A
G3679-1	1355360	1349519	1897	3944	0.29	N/A
G3680-1	149784	149675	13	96	0.06	N/A

G3682-1	3504931	3502225	615	2091	0.06	N/A
G3683-1	1207038	1202736	1261	3041	0.25	N/A
G3686-1	4507006	4505341	559	1106	0.02	N/A
G3687-1	2186658	2176377	4371	5910	0.27	N/A
G3729	2759764	2757795	642	1327	0.05	N/A
G3734-1	838143	822305	7469	8369	1.00	N/A
G3750-1	837699	824731	5673	7295	0.87	N/A
G3750-2	400166	389304	3970	6892	1.72	N/A
G3750-3	341177	330270	4533	6374	1.87	N/A
G3758	1844108	1841345	1261	1502	0.08	N/A
G3771	341011	337765	968	2278	0.67	N/A
G3782	1200724	1197578	599	2547	0.21	N/A
G3786	358637	348679	2455	7503	2.09	N/A
G3788	1334836	1320139	5732	8965	0.67	N/A
G3841	971191	957371	6507	7313	0.75	N/A

Table 2: EBOV and bacterial load in 4 evolution points of the Ebola disease in a discharged patient

Evolution Points	EBOV copies/ml	% bacterial reads	Outcome
G3769-1	1.12E+06	0.539633236	Discharged
G3769-2	4.10E+06	0.597289625	Discharged
G3769-3	3.71E+05	0.998270488	Discharged
G3769-4	1.60E+07	0.035288415	Discharged

Figure 3: EBOV and bacterial load in 4 evolution points of the Ebola disease in a discharged patient
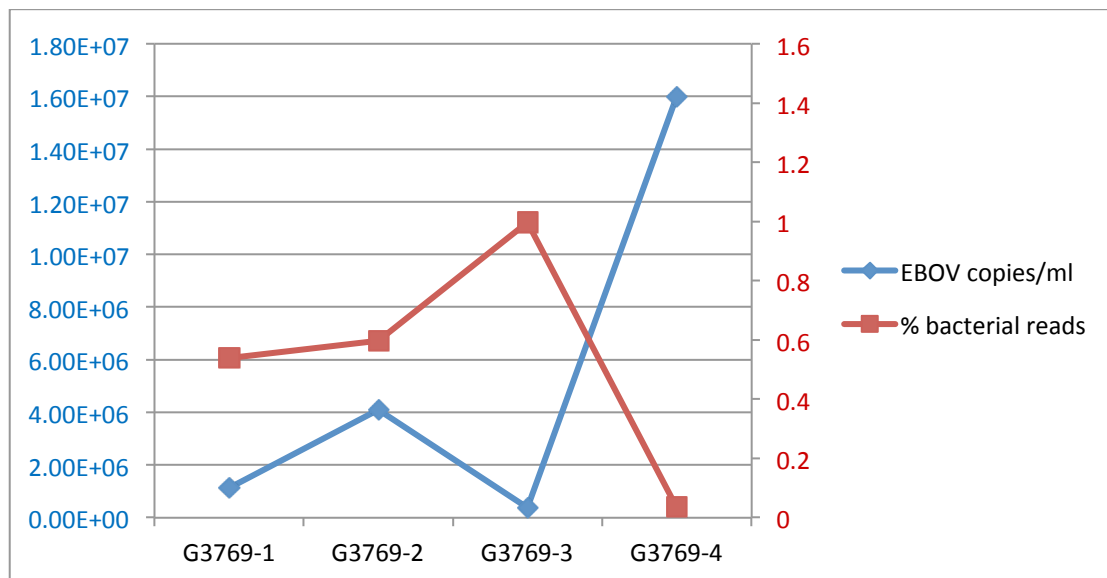
Table 3: EBOV and bacterial load in 4 evolution points of the Ebola disease in a patient with fatal outcome

Evolution points	EBOV copies/ml	% bacterial reads	Outcome
EM124-1	3.11E+06	0.171251123	Died
EM124-2	1.45E+05	1.41980041	Died
EM124-3	2.07E+04	0.37457228	Died
EM124-4	7.44E+03	0.389441022	Died

Figure 4: EBOV and bacterial load in 4 evolution points of the Ebola disease in a died patient

Figure 1: Percentage of the top 30 most abundant bacterial detected in 99 RNA-Seq samples[4] from Ebola patient serum using the Best Blast Hit paradigm of taxonomic assignment (the frequencies are not cumulative)

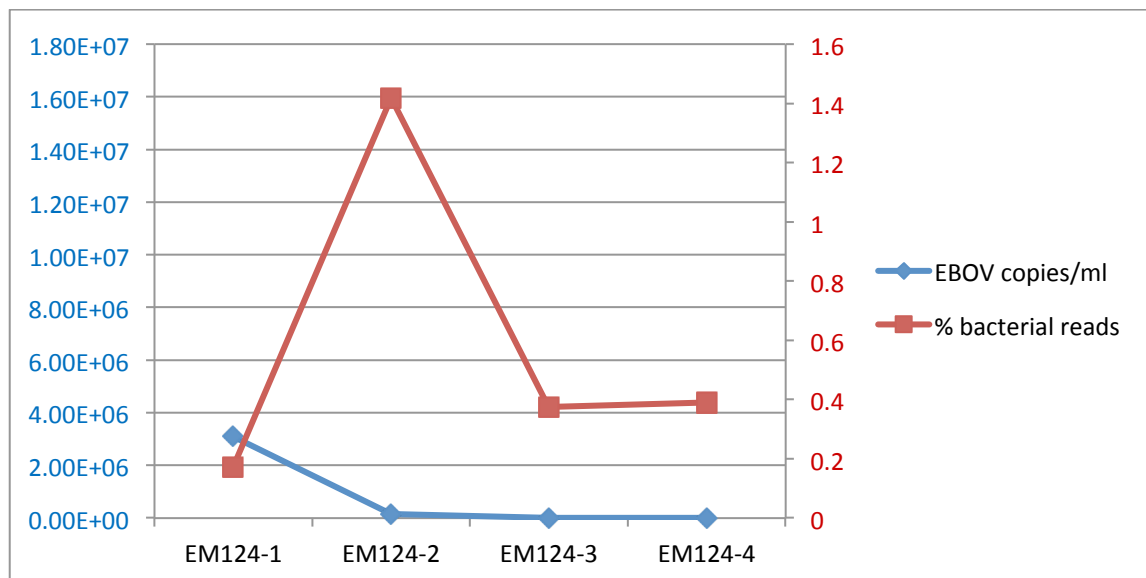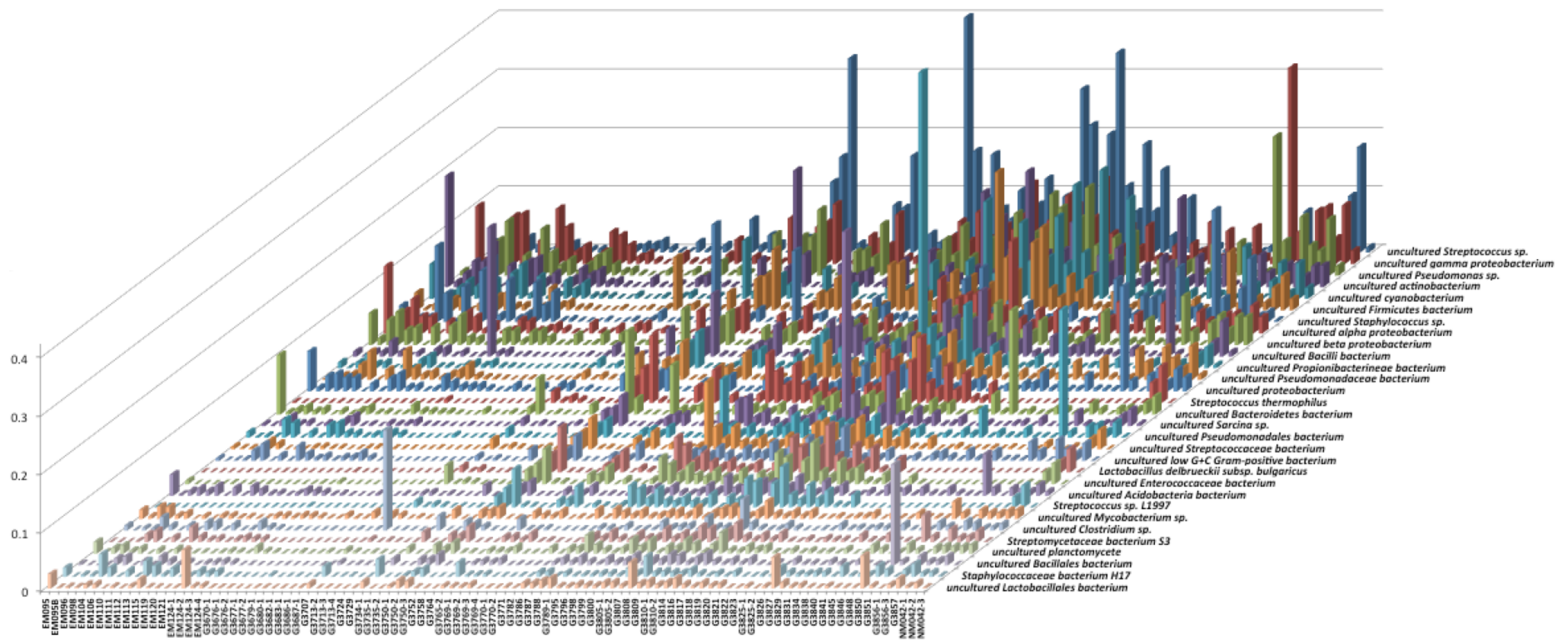**Figure 2:** Percentage of the top 30 most abundant bacterial detected in 99 RNA-Seq samples[4] from Ebola patient serum using the Lowest Common Ancestor paradigm of taxonomic assignment (the frequencies are not cumulative)

uncultured Streptococcus sp.
uncultured gamma proteobacterium
uncultured Pseudomonas sp.
uncultured actinobacterium
uncultured cyanobacterium
uncultured Firmicutes bacterium
uncultured Staphylococcus sp.
uncultured alpha proteobacterium
uncultured beta proteobacterium
uncultured Bacilli bacterium
uncultured Propionibacterineae bacterium
uncultured Pseudomonadaceae bacterium
uncultured proteobacterium
Streptococcus thermophilus
uncultured Bacteroidetes bacterium
uncultured Sarcina sp.
uncultured Pseudomonadales bacterium
uncultured Streptococcaceae bacterium
uncultured low G+C Gram-positive bacterium
Lactobacillus delbrueckii subsp. bulgaricus
uncultured Enterococcaceae bacterium
uncultured Acidobacteria bacterium
Streptococcus sp. L1997
uncultured Mycobacterium sp.
uncultured Clostridium sp.
Streptomycetaceae bacterium S3
uncultured planctomycete
uncultured Bacillales bacterium
Staphylococcaceae bacterium H17
uncultured Lactobacillales bacterium

Bacteria
Firmicutes
Proteobacteria
Actinobacteria
Streptococcus
Alphaproteobacteria
Bacilli
Betaproteobacteria
Bacteroidetes
Lactobacillales
Cyanobacteria
Staphylococcus
Lactobacillus delbrueckii
Lactobacillus
Gammaproteobacteria
Bacillales
uncultured cyanobacterium
Actinomycetales
Rhodococcus
Carnobacterium
Micrococcineae
Methylobacterium
Leuconostoc
Clostridiales
Streptococcaceae
Aerococcus
Paenibacillus
Fusobacteria
Pseudomonas
Lactobacillaceae

Plano inferior