Microbial Pathogenesis 105 (2017) 231–234

Contents lists available at ScienceDirect

Microbial Pathogenesis

journal homepage: www.elsevier.com/locate/micpath

Should we care about transmission of infectious bacterial agents through serum handling?

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ARTICLE INFO

Article history:
Received 25 February 2016
Received in revised form
10 January 2017
Accepted 23 February 2017
Available online 24 February 2017

Keywords:
Serum
Infectious bacterial agent
Transmission

ABSTRACT

Immunological assays are valuable diagnostic tools to detect infections due to most of bacterial microorganisms. However, the question is how much safe are the common serum samples used as sources of antibodies in these assays? Here, we experimentally followed the issue. 10-fold serial dilutions of two Gram-positive, *Staphylococcus aureus* and *Bacillus cereus*, and one Gram-negative bacteria, *Escherichia coli*, were prepared and spiked into freshly taken blood samples of human and four animal species including sheep, goat, cattle, and horse. After blood clotting, serum samples were examined by colony count method before and after a centrifugation step followed by an observation with a scanning electron microscope. No bacteria grew from both centrifuged and non-centrifuged serum samples contained at least 7.5×10^2, 7.5×10^5, and 7.5×10^5 CFU/ml of *S. aureus*, *B. cereus*, and *E. coli*, respectively. Moreover, routine centrifugation criteria did not show any significant effect on decrease of bacterial cells in sera. The results suggest that we can handle serum samples of apparently healthy humans and animal species without deep concern for possibility of transmission of infectious bacterial agents. However, this supposition should not completely be excluded.

1. Introduction

Every laboratory has a limited authority to work with distinct biologically dangerous infectious agents which is determined by its biosafety level [1]. This does not necessarily mean that diagnostic or research laboratories cannot carry out diagnostic serological experiments, as these assays are useful methods for characterization and identification of various infections caused by infectious agents including bacteria, viruses, parasites, etc. However, it should be considered that some serum samples belong to cases of bacteremia, either common cases or immunocompromised patients [2–5] and it has been shown that there are different infectious bacterial species which can cause such a fatal bacteremia or septicemia [6–10]. Thus, it should experimentally be examined if serum samples are potential sources of infectious bacterial agents or not, in particular, for those bacteria with zoonotic importance. This includes two types of prepared serum samples, those which are normally prepared by simple blood clotting without centrifugation and those which are procedurally prepared by blood clotting followed by centrifugation as recommended [11]. Therefore, the present study was conducted to find out whether infectious bacterial cells are present in serum samples or not. Because, handling of such serum samples may lead to the transmission of infectious organisms to laboratory operators.

2. Materials and methods

2.1. Bacterial cells

Two Gram-positive bacteria, *Staphylococcus aureus* ATCC 6538 and *Bacillus cereus* ATCC 11778, and one Gram-negative bacterium, *Escherichia coli* ATCC 35218 were used in the present experiment. These strains were chosen based on the fact that they have different physico-chemical characteristics and bloodstream infections due to these bacteria have been previously described [5–8]. *S. aureus* (coccus) and *B. cereus* (rod) are bacteria with an average diameter of

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http://dx.doi.org/10.1016/j.micpath.2017.02.037
0882-4010/

0.8–1 μm and 1 × 3–5 μm, respectively and *E. coli* is a medium-sized rod with a diameter of 0.4–0.6 × 2–3 μm [12].

2.2. Bacterial suspensions

A 0.5 McFarland standard (~1.5 × 10^8 CFU/ml) bacterial cell suspension of each of the bacterial species was prepared in phosphate buffered saline (PBS) from an overnight (18 h) nutrient broth culture as a stock solution. Afterward, 10-fold serial dilutions of bacterial suspensions were prepared in PBS from each of the stock solutions and used to spike freshly taken blood samples. All of the solutions were prepared in the same day of blood collection.

2.3. Collecting of serum samples

After blood sampling from human and four animal species including sheep, goat, cattle, and horse, 500 μl of each of the freshly taken whole blood samples was immediately added to a microtube contained the same volume of each of the bacterial dilutions and mixed gently by inverting several times. The microtubes were then incubated at 37 °C for about 1.5–2 h to allow blood clotting.

2.4. Counting of bacterial cells

50 μl of each of the collected serum samples (non-centrifuged serum samples) was examined in duplicate using colony count method. Thereafter, the microtubes were centrifuged at 1500 × g for 10 min and once again, 50 μl of each of these serum samples (centrifuged serum samples) were cultured in duplicate on sheep blood agar to investigate the effect of centrifugation on the number of present bacterial cells in the sera. Finally, the mean number of grown colonies was measured as CFU/ml. A serum sample was also collected from each of the stock blood samples as a negative control and examined to see if the blood samples had been already infected with bacteria.

2.5. Scanning electron microscope (SEM)

To confirm entrapment of bacterial cells in fibrin networks, a corresponding sample of each of the tested bacterial species was prepared and observed under a scanning electron microscope. Briefly, the clot samples were fixed in 2.5% buffered glutaraldehyde followed by dehydration in graded ethanol. The samples were then dried at room temperature and glued onto stubs. Finally, the processed samples were sputter-coated with gold and examined in a JEOL JSM-840 scanning electron microscope operating at an accelerating voltage of 15 kV.

2.6. Statistical analysis

According to the initial concentration of the bacterial stock solutions (~1.5 × 10^8 CFU/ml), paired-samples T test was performed to test the significance of differences between measured log CFU values of various dilutions before and after clot formation for each examined samples using a SPSS software (version 16.0). To accomplish the statistical test, the number of grown colonies for sera with no growth assumed to be 1 CFU/ml. The comparison was also done for centrifuged and non-centrifuged sera.

3. Results

The mean number of colonies which grew from various serum samples was different for each bacterial species and statistical analysis showed that there were significant differences between measured log CFU values for differentially spiked bacteria before and after clot formation ($P < 0.01$). Considering the minimum

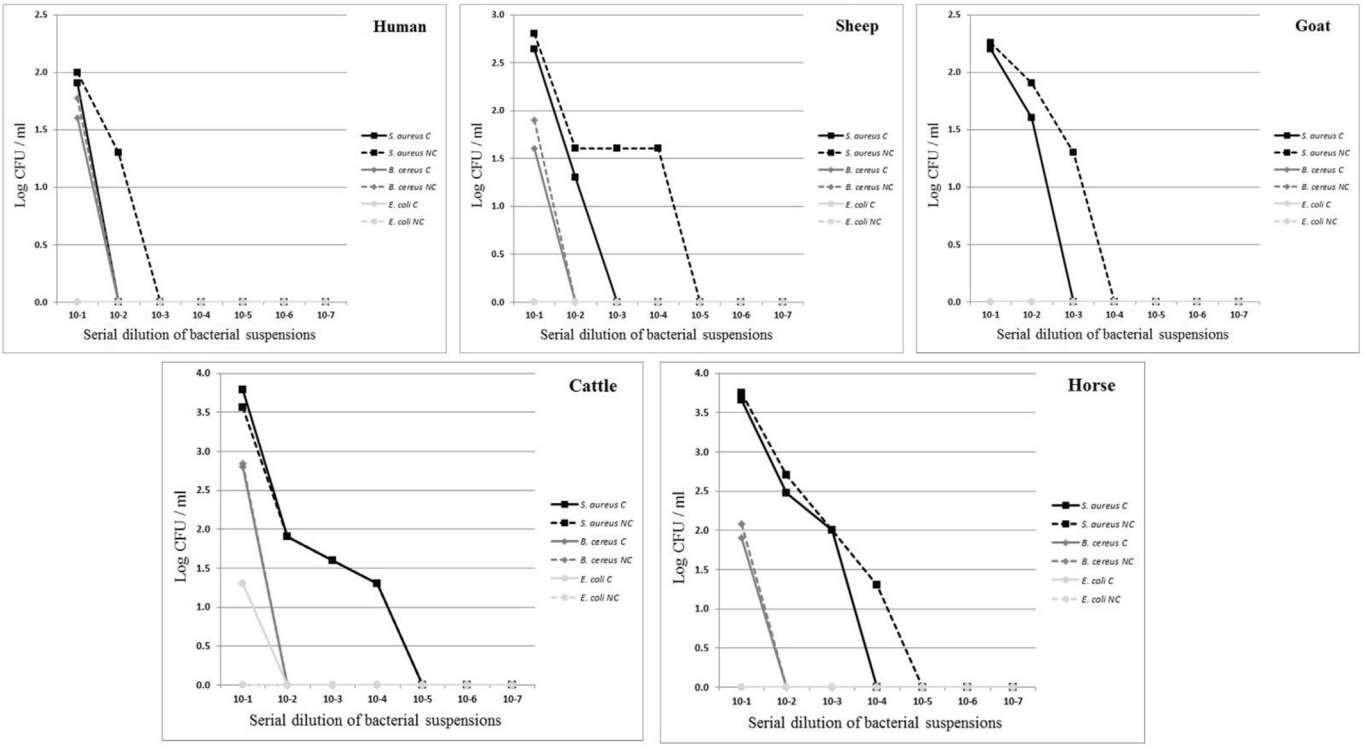

Fig. 1. Log mean number of grown colonies from cultured serum samples spiked with serial dilutions of bacteria (serial dilutions: 10^{-1} to 10^{-7}, NC: non-centrifuged, C: centrifuged serum samples).

dilution from which no bacteria grew, it seems that the most fibrin-entrapped bacteria were *E. coli* cells, while the most bacterial cells which fled from fibrin networks were *S. aureus* cells. However, size of the latter species is smaller than *E. coli* cells. Besides, no bacteria grew from serum samples which had not been spiked with bacterial cells (negative controls).

Fig. 1 shows log CFU/ml values for different bacterial cells in various species. No significant difference ($P > 0.05$) was observed between the numbers of grown bacterial colonies from centrifuged and non-centrifuged serum samples.

Totally, as 500 μl of each of the bacterial dilutions had been mixed with 500 μl of blood samples, the maximum concentrations from which no bacteria grew, contained at least ~7.5×10^2 (= ~$1.5 \times 10^8 \times 10^{-5}/2$), 7.5×10^5, and 7.5×10^5 CFU/ml for *S. aureus*, *B. cereus* and *E. coli*, respectively. This for example means that even 7.5×10^5 *E. coli* bacterial cells per 1 ml of blood could be precipitated by the process of clot formation. The minimum dilution for each bacterial suspension from which no bacteria grew are presented in Table 1.

Meanwhile, to provide supporting evidences to indicate entrapped bacteria during clot formation, clot samples were also inspected using a SEM. Such tangled bacterial cells are illustrated in the Fig. 2. As shown, the spiked bacterial cells were well entrapped in fibrin networks while surrounded by some RBCs and other blood components.

Table 1
The minimum dilution for each of the bacterial suspensions from which no bacteria grew.

Blood	*S. aureus*	*B. cereus*	*E. coli*
Human	10^{-3}	10^{-2}	10^{-1}
Sheep	10^{-5}	10^{-2}	10^{-1}
Goat	10^{-4}	10^{-1}	10^{-1}
Cattle	10^{-5}	10^{-2}	10^{-2}
Horse	10^{-5}	10^{-2}	10^{-1}

4. Discussion

Serological and bacteriological laboratories usually perform common serological assays to diagnose bacterial infections using serum samples of suspected human and animal hosts. It is absolutely important to know how much safe such samples are. This can be expected that phagocytosed bacterial cells will be precipitated during clot formation. However, the story may be different for bacterial cells freely circulating in the blood stream. Escaping of these bacterial cells from fibrin-mediated physical entrapment and their presence in the serum samples may result in possible transmission to laboratory operators through serum handling. In the present study, it was shown that the process of blood clot formation could effectively remove bacterial cells of different sizes (at least 7.5×10^2 CFU/ml) from non-centrifuged sera independent to bacterial and host species. In spite of the fact that *E. coli* cells are almost smaller than bacilli; the most precipitated bacterium in this process was *E. coli* for all types of the tested bloods. Indeed, only 20 CFU/ml grew from a cattle serum sample spiked with 10^{-1} dilution of *E. coli*. This could likely be due to the different cell wall properties of this Gram-negative bacterium indicating that the size of the bacterial cells may not be the only factor responsible for the precipitation of bacteria by fibrin networks. Meanwhile, possible reaction of anti-bacterial antibodies which may be present in the serum samples should also be considered. However, as a control, the bacterial suspensions were also spiked into the collected serum samples and incubated for the same time (equal time for clotting) to investigate any anti-bacterial activity of the sera. The results were completely different and too many colonies grew from serum samples in this

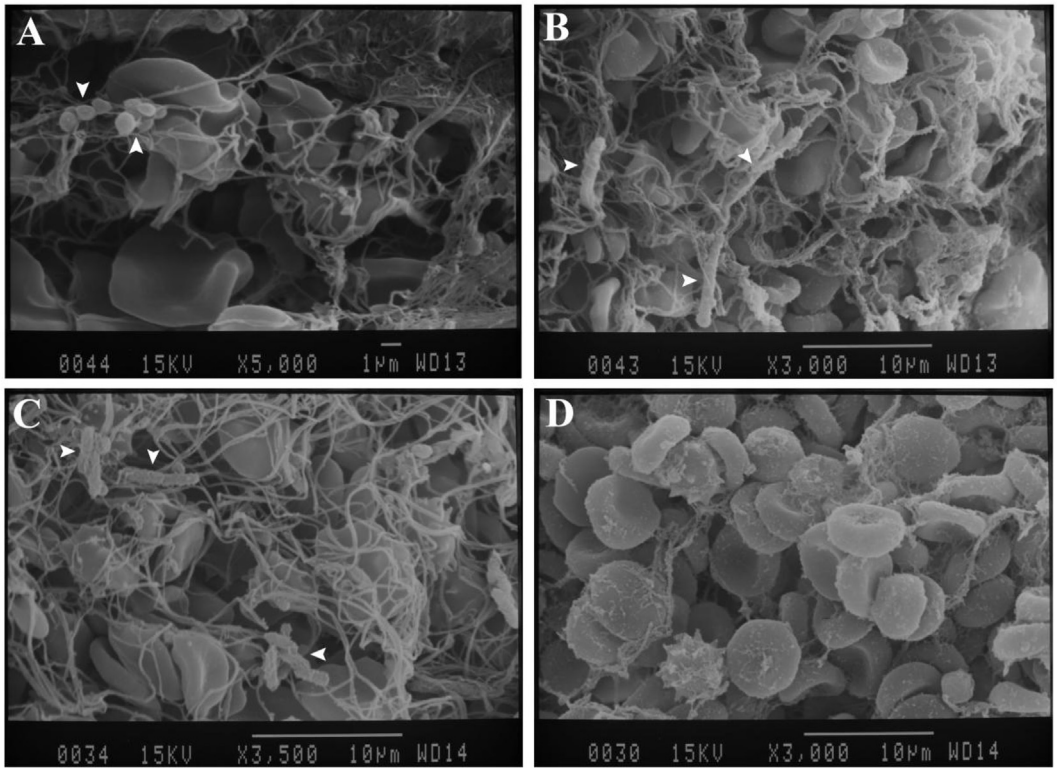

Fig. 2. Entrapped bacterial cells (arrow heads) of three different species (A: *S. aureus*, B: *B. cereus*, C: *E. coli*, and D: negative control contained no bacteria) in the fibrin networks. All clot samples were prepared from a human blood sample which had been separately spiked with the prepared 0.5 McFarland solutions of the bacteria.

case (data not shown). Moreover, despite that *S. aureus* was the smallest tested bacterium, the maximum number of grown colonies belonged to it, whereas, a similar reaction was not observed for the other two bacterial species indicating diverse reactivity between the serum samples and bacterial species. Meanwhile, there were no significant differences ($P > 0.05$) between the centrifuged and non-centrifuged serum samples in the number of grown bacterial colonies (Fig. 1) suggesting that common centrifugation criteria may only be effective to obtain a more clear serum sample free from debris and clots. It should be noticed that generally, the number of bacterial cells in the serum samples from which no bacteria grew, still seems to be higher than the usual viable colony count expecting from a conventional blood culture of a case with bacteremia [13]. Nevertheless, growth of bacterial colonies from the blood samples spiked with high concentrations of bacteria suggests that there is still a chance of presence of bacterial cells in serum samples taken from peracute and acute septicemic cases and precautions should be considered for working with them. For instance, it was reported that the number of *Bacillus anthracis* cells in a susceptible animal's blood approaches death may be as many as 10^9 CFU/ml (depending on the species), which is higher than the maximum concentration of tested bacteria in the present experiment, and this will increase the probability of presence of bacterial cells in a serum sample collected from such an animal [14]. Totally, it appears that several factors including number, shape, and size of bacterial cells, tendency for aggregation and clump formation, and physico-chemical properties of the bacterial cell walls may likely contribute to the possibility of presence of bacterial cells in serum samples. However their different potential roles should be investigated.

5. Conclusions

The results of the present study suggest that as the numbers of the examined bacterial cells seem to be higher than viable colony count expecting from blood cultures of most usual cases of bacteremia or septicemia, we can handle normal serum samples of apparently healthy humans and animal species without deep concern for possibility of transmission of infectious bacterial agents. However, as there were also grown bacterial colonies from samples contained high concentrations of bacterial cells; this assumption should not completely be excluded. Knowing this is very important, especially when there is possibility of presence of zoonotic infectious bacterial agents in serum samples.

Conflict of interest statement

Authors declare that there is no conflict of interest.

Acknowledgements

We thank Iraj Badri and Mostafa Rezaei who helped us in collecting blood samples. This project was funded by research grants (No 95-203) from Bu-Ali Sina University, Hamedan, Iran.

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