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Molecular analysis of cross-bacterial contamination detected in biotin-free buffers during diagnosis of HCV infections

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ABSTRACT: In a routine work to determine Hepatitis C Virus (HCV) molecules in human infected serum with biotin/streptavidine enzyme linked immunosorbant assay (ELISA) technique, unexpected false positive was observed. No positive signals were noticed after changing all ELISA buffers. Subsequently, contaminated buffers were screened and analyzed for microbial contamination. Out of fifty-five, five randomly selected bacterial colonies were examined for biosynthesis of biotin using ELISA and/or Western blot binding biotin techniques in both supernatants and cell pellets. In compare to the *E.coli* reference strain a strong biotin signals in all examined isolates were recorded. All isolates were then examined for their genetic heterogeneity by PCR-RFLP technique of 23S rDNA genes. While, isolate BP(R2) which gave the highest biotin signal, was subjected for molecular identification. Comparative sequence analysis of the 16S rDNA gene (~1440 bp) revealed that this isolate is a member of the bacterial genus *Delftia* exhibiting a similarity value of 99.3% with *Delftia acidovorans*. In conclusion, a soluble biotin is secreted by the isolate *Delftia acidovorans* BP(R2) and it is also coupled to protein with molecular weight 25-26 KDa. As well as, this bacterial contamination was the reason for the false positive results observed during the detection of HCV infections. *@JASEM*

Hepatitis C virus (HCV) is a one of the major causes of chronic hepatitis that can lead to liver cancer (Aoyagi, et al. 1999) and its infection occurs among persons of all ages, but the highest incidence of acute hepatitis C is found among persons aged 20-39 years, and males predominate slightly (Alter, et al. 1990). It is known that this pathogen infects people through various ways include; blood transfusion, injecting-drug use, employment in patient care or clinical laboratory work, exposure to a sex partner or household member who has had a history of hepatitis, exposure to multiple sex partners, and low socioeconomic level. However, Alter, et al. (1982) reported that no association with military service or exposures resulting from medical, surgical, or dental procedures, tattooing, acupuncture, ear piercing, or foreign travel. If transmission from such exposures does occur, the frequency might be too low to detect Alter, et al. (1989).

To control viral diseases, blood test is crucially important in clinical analysis and blood products. The diagnosis of HCV infection can be made by qualitatively detecting HCV RNA using gene amplification techniques (e.g., RT-PCR) (Gretch, et al. 1995). HCV RNA can be detected in serum or plasma within 1-2 weeks after exposure to the virus and weeks before the onset of alanine aminotransferase (ALT) elevations or the appearance of anti-HCV. The only tests currently approved by the U.S. Food and Drug Administration (FDA) for diagnosis of HCV infection are those that measure anti-HCV (Gretch, 1997). These tests detect anti-HCV in greater than or equal to 97% of infected patients, but do not distinguish between acute, chronic, or resolved infection. In addition, one of the most common current methods for the detection of infected blood samples is biotin/streptavidine enzyme linked immunosorbant assay (ELISA) that detects the presence of anti-HCV in the serum (Bouvier-Alias et al. 2002). Basically, Avidin is a glycoprotein derived from egg albumin, which has a very high affinity for the vitamin, biotin, and does not bind to other substrates. Biotin can be easily coupled to IgG antibody. Avidin can be labeled with fluorochromes, enzymes, or radioactive chemicals. If the secondary antibody are labeled, using the biotin-avidin system, they can be efficiently employed in indirect IF, ELISA and RIA. This technique is highly sensitive, but may yield false negative and/or false positive results due to cross-reactivity with other antigens or microbial contamination.

In the present study, during a routine-work to detect HCV infection with biotin/streptavidine ELISA, unexpected false positive results were observed. Subsequently, the major goal of this study was to put the hand on the right reason of these unexpected false positives. To achieve this target, two different strategies were followed: the first was to change the coating materials and affinity binding lectins with different kinds of blocking agents, while, in the second, it was supposed a microbial contamination may be the source of these false positives.

MATERIALS AND METHODS

Enzyme linked immunosorbant assay (ELISA): The microtiter plates (Corning-Costar, UK) were coated with 1µg/100ul/well of egg avidin (purified from

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eggs according to DeLange, 1970), in 0.05 M carbonate/bicarbonate buffer (pH 9.6) then incubated for 3 h at room temperature followed by overnight incubation at 4^{o}C. They were used over test period of 2 month without loss of sensitivity. After the incubation, the plates were washed with PBS (pH 7.2) containing 0.05% Tween 20 then blocked with (1% BSA, 5% skim milk, 1% gelatin, 1% casein, or intravenous IgG product)-0.1% Tween 20 in PBS for 1 h at 37^{o}C. The diluted sample (biotin) in PBS-0.05% Tween 20) were distributed (100 µl/well) and incubated for 1 h at 37^{o}C. After incubation, peroxidase-labelled streptavidine (Sigma, MO, USA) diluted 1:5000 in PBS-Tween 20 buffer was added. Plates were incubated for 1 h at 37^{o}C washed with PBS-Tween 20, then with PBS alone. The ready made 3,3',5,5'-Tetramethylbenzidine (TMB) substrate (Sigma) was added to the plates. The reaction was terminated after 10 min by adding 0.5 M HCl.

Isolation of bacteria:An aliquot 100µl of each contaminated buffer was taken and plated onto minimal salts agar plates (MSM) containing per liter: 2.75g of K_2HPO_4, 2.25g of KH_2PO_4, 1.0g of $(NH_4)_2SO_4$, 0.2 g of $MgCl_2$ $6H_2O$, 0.1g of NaCl, 0.02g of $FeCl_3$ $6H_2O$, and 0.01g of $CaCl_2$ (pH 6.8 to 7.0) supplemented with 2.5% (w/v) glucose as carbon and energy source. Colonies were apparent after incubation for 24 h at 37°C. Subsequently, five single colonies were selected for further studies. *E. coli* DH5-α F (Invitrogen, USA) was used as a reference strain. Pure cultures were stored in 50 mM KH_2PO_4 (pH 7.2) containing 20% (v/v) glycerol at -70°C till used.

*Biotin determination of the selected isolates:*Single colony of the randomly selected isolates was picked in 3 ml MSM or LB (contained (g/l) 5 yeast extract, 10 casein peptone, 10 NaCl, pH 7.0) media then shaken at 37°C for 24 h. Subsequently, 1 ml of the culture was transfer to an Eppendorf tube and centrifuged at the maximum speed for 10 min. The cell free supernatant was subjected for Biotin determination using the ELISA assay; while the cell pellet was save for SDS-PAGE/Western blot analysis (Redwan, 2002).

SDS-PAGE and Western blotting: Electrophoretic separations of bacterial proteins were performed under reducing conditions using 12.5% gels (Laemmli, 1970). Two hundreds microlitter of bacterial culture at 0.6 (600 nm) was centrifuged and the pellet mixed with 200uL sample buffer, and 50 uL of sample was applied to each lane. Following SDS-PAGE, gels were transblotted or stained with Coomassie brilliant blue R-250 (BioRad, CA, USA). The bacterial proteins separated by SDS-PAGE were transblotted onto nitrocellulose sheets. The blot was blocked for 60 min in 3% BSA in 0.05% Tween 20-Tris buffer saline (TBS), then washed three times (5-10 min/each) with TBS (0.5% BSA-0.1%Tween 20). The blot was incubated with streptavidin-peroxidase labeled (1:5000) for 2 hours at room temperature. After washing, the blot was visualized with ready made 4-Chloro-1-Naphathol substrate (Sigma).

PCR-RFLP of 23S rDNA genes: To determine the genetic heterogeneity among the isolates, rapid identification scheme based on PCR restriction fragment length polymorphism (PCR-RFLP) analysis of the 23S rDNA genes was performed (Abd-El-Haleem et al. 2002). Universal bacterial PCR primers specific to amplify ~2700 bp of 23S rDNA gene were used (Terefework et al., 1998). For restriction endonuclease digestion a 20µl reaction mixture that included 10 µl of the PCR amplicon with 5U of the restriction endonuclease *Sal*I and *Hind* III was employed, following conditions recommended by the respective manufacturers (Gibco BRL, USA). Upon completion of the reaction, an aliquot 10 µl was analyzed by electrophoresis in a 2% agarose gel in TRIS-acetate-EDTA buffer (pH 8.5). The gel was stained with ethidium bromide (0.5 µg/ml: Sigma, Bornem, Belgium) and then visualized and photographed in MultiImage light cabinet (Alpha Innotech Corporation, USA). The sizes of the amplified products were assessed by comparison with a commercial weight marker (Invitrogen, Netherlands).

Sequence analysis of 16S rDNA: Molecular identification of isolate BP(R2) that gave the highest biotin signal among all tested isolates was performed. Complete sequencing was done by the amplification of 16S rDNA with bacterial universal primers 27F and 1492R (Ludwig and Schleifer 1994). For sequencing PCR products were purified using Qiaquick spin columns (Qiagen, USA). Sequencing was performed by the Core laboratory facility of the University of Tennessee (Knoxville, USA). The sequence was analyzed using the BLAST program (National Center for Biotechnology Information) to determine the closest available database sequences. Selected DNA sequences were aligned using the Clustal W program (Altschul et al. 1990). Published sequences were obtained from GenBank. A phylogenetic tree was constructed using Clustal W by distance matrix analysis and the neighbour -joining method (Saitou and Nei 1987). Bootstrap analysis was used to provide statistical confidence for the tree branch points. Phylogenetic trees were displayed using TREEVIEW (Page 1996).

RESULTS AND DISCUSSION

Rabbit polyclonal antibody against five synthetic peptides of core and envelope HCV was used as coating material to capture the denaturized HCV antigens from infected patients' serum. The coupled antibody-peptides-biotin was used as a second antibody to detect the first antibody, then streptavidin-peroxidase was added to find the antibody-biotin complex. ELISA-plate rows omitted the HCV antigens and/or antibody-biotin steps raise a very strong signal denoted to biotin binding. The same results were obtained by repeating the assay three times. Subsequently, to solve and analyze this problem, parameters such as coating materials, kind of blocking agents, and prepare a new stock from anti-peptides free biotin or coupled to biotin were analyzed.

Coaster plates (96 wells) were coated (except the first column, used as blank) with purified Egg Avidin (10µg/ml). After vigorously washing the plates were blocked with 200uL of 1% Bovine Serum Albumin BSA-, 3% Skim Milk- (S.M.), or 1% Casein-0.1%Tween 20. 100µL of the contaminated sample was added to each test wells panel, the control well left empty. The plates were process as a standard ELISA assay as mention under material and methods part. The results presented in Table 1 show the differences between blocking agents (BSA, Skim Milk-, Casein-and IVIG-Tween 20). The minimal background was recorded in BSA-Tween 20, subsequently it was used throughout the study. Gelatin and IVIG results were excluded because they are not reproducible and gave a strong background (data not shown). In addition, these results indicate a strong biotin source may be present in this buffer, and obviously excluded the coating buffer, blocking agents, first and second antibody as a source of the biotin. In addition, these results indicate a strong biotin producer may be present in this buffer, and obviously excluded the blocking agents as a source of the omitted biotin.

Table 1: Clarifying the biotin in the contaminated buffer and the effect of the blocking agents.

Coating agent		EggAvidin (1ug/well)			
Blocking agent		BSA/Tween20	S.M./Tween20	Casein/Tween20	Positive control
Biotin source		Control^{+} : test	control : test	control : test	control: test*
Streptavidin-HRP		100uL/well of 1:5000 streptAvidin			
Substrate		100uL/well of TMB			
OD 450nm	Control	0.08±0.003**	0.101±0.013	0.081±0.006	0.080±0.009
	Test	1.05±0.080	0.957±0.096	1.090±0.023	1.590±0.298

+The control wells were omitted the biotin source (as contaminated buffer or pure).

- Positive control has 10nm/ml of pure biotin in PBS, ** All results are represented as a mean of 11 wells/control or test ± standard deviation after subtraction the blank values (8 wells).

Consequently, the contaminated buffer was subjected for isolating pure microbial colonies by plating on minimal salts agar plates. Five single grown colonies named BP1, BP(R2), BP3, BP4 and BP5 were randomly selected. Comparing to the *E. coli* reference strain, a significant biotin signals ($P < 0.005$) in the cell-free supernatant of all tested isolates were recorded. However, the highest biotin yield was observed in isolate BP(R2), which then used in the further studies and the rest of isolates were excluded. No significant difference in the biotin signals due to the use of LB or MSM media were observed (Table 2). These results supported the idea that profusion of soluble biotin is secreted by bacterial isolate.

Table 2: Assessment of biotin in both LB and MSM media.

Coating agent	EggAvidin		Negative	Positive
Blocking agent	1%BSA-0.1% Tween20			
Biotin source from	100uL LB*	100uL of MSM**	Supernatant of E.coli	10nm pure biotin/ml
StreptAvidin-HRP	100uL of 1:5000 StreptAvidin			
Substrate	100uL of TMB			
OD 450 nm	0.590±0.203	0.890±0.033	0.185±0.015	1.59±0.290

ELISA assay was performed as in table 1, except the biotin source was from the microbial growth in both essential and normal medium, in addition of *E.coli* DH5-α F as control strain. The plates were blocked with 1%BS-0.1% Tween 20 (the best blocking agent). *LB broth: Luria-Bertani Medium, **MSM: Minimal Salts Medium.

To clarify if this biotin is free or coupled to protein, dot-ELISA (The proteinase K treated samples) and Western blot techniques were performed. The data presented in both Fig 1 and Table 2 revealed signals for binding of streptavidin-peroxidase to biotin in both techniques. These results demonstrated that the biotin is coupled to protein either in the bacterial isolates or the *E. coli* reference strain, while it was secreted in our isolate only. The signal came from BP(R2) strain was more intense than *E. coli* signal on the Western blot and the protein containing biotin has a molecular weight 25-26 KDa.

Morphologically, all five selected isolates have a similar colony shape and color, positive in Gram stain and their antibiotic resistant profile showed only resistant with ampiciline (50mg/l) and failed with kanamycine (50mg/l) or tetracycline (10 mg/l). They are also growing well in incubation temperature ranged between 4°C and 40°C for 24 and 48 h, respectively. Restriction fragment length polymorphisms (RFLP) of the 23S rDNA PCR amplified products with endonucleases enzymes *Sal*I and *Hind*III produced clearly distinguishable patterns. The reference strain *E. coli* DH5-α F could be recognized from the isolates by any one of the two restriction enzymes (Fig. 2A and 2B). As shown in Fig 2A the *Sal*I generated RFLP patterns success to reach a conclusion that the isolates BP1, BP2 (R2), BP3, BP4 and BP5 are not *E. coli* related strains and it failed to discriminate among all isolates (data not shown). However, enzyme *Hind*III classified them to two groups: the first includes isolates BP1, BP2(R2), BP3 and BP4, while the second includes only isolate BP5 (Fig. 2b).

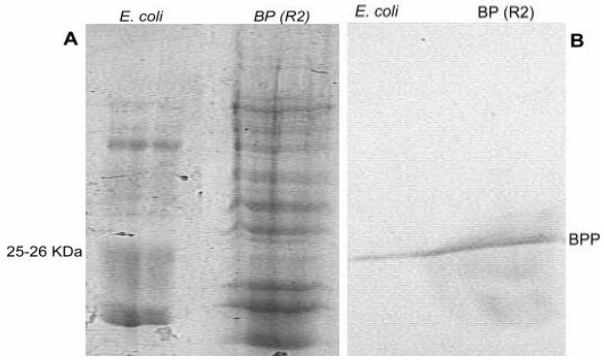

Fig 1. A culture aliquot (200uL) was centrifuged and the cells pellet was solublized in 1X sample buffer, then loaded onto 12.5% SDS-PAGE. All samples were boiled 10 minutes prior to loading; the gel was stained with Coomassie blue R250 (A) or transblotted into nitrocellulose sheet, and then stained with ExtraAvidin-Peroxidase (1:5000) biotin staining kit (B).

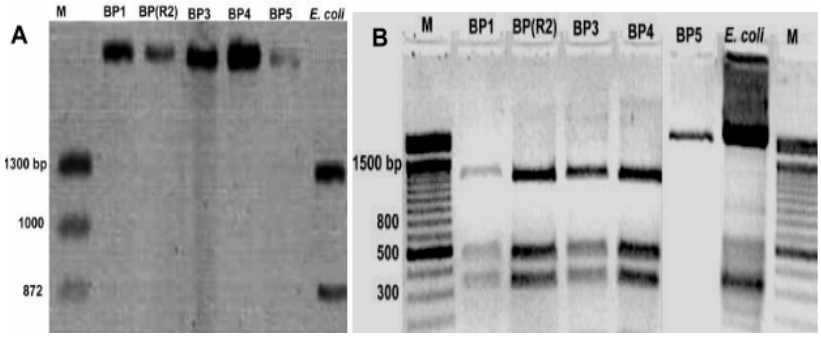

Fig 2. PCR-RFLP patterns of 23S rDNA genes observed with both enzymes *Sal*I (Fig 2A) and *Hind*III (Fig 2B), lanes 2, 3, 4, 5, 6 and 7 are the patterns for the isolates BP1, BP2 (R2), BP3, BP4, BP5 and *E. coli* DH5-α F, respectively. Lane M, DNA ladder. BPP, 25-26KDa, and BP on figure sides pointed to biotin binding protein, the estimated molecular wt and biotin-producing bacterial isolate, respectively.

Comparative sequence analysis of 16S rDNA gene (~1440 bp) of isolate BP2(R2) that gave the highest biotin yield, reveled that this isolate is a member of genus *Delftia* exhibiting similarity values 99.3% to *Delftia acidovorans* (Fig 3). The nucleotide sequence of this isolate has been deposited in the NCBI nucleotide sequence databases (GenBank) under accession number of AF410435.

Microorganisms such as *Escherichia coli*, *Bacillus subtiles*, *Marcescens*, *Sinorhizobium meliloti* and *Kurthia* have been previously reported as biotin producers (Hofmann et al 2000, Kiyasu 2001). For our knowledge this is the first report mention that one of *Delftia acidovorans* genus might synthesis a free and coupled biotin. These results are synochronized with the previous studies which indicate that the biotin is synthesized in both Gram-positive and Gram-negative bacteria by a well-defined pathways (Marquet, 2001). In gram-positive bacteria, the first steps of biotin biosynthesis require the *bioW* and *bioI* genes, and in Gram-negative microbes, the *bioC* and

bioH genes are required for this step. The last step requires *bioB* and *bioY* in Gram-negative and positive microbes, respectively (Sullivan et al. 2001). The analytical analysis of free biotin using HPLC and Mas-sectrophoometry, and the molecular cloning of its gene(s) to identify the pathways of synthesis by isolated *Delftia acidovorans* strain BP(R2) are currently underway.

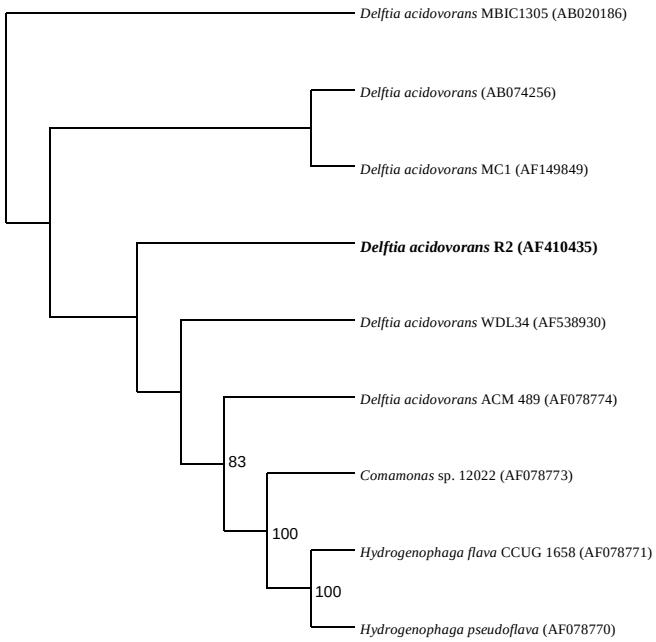

Fig 3: Phylogenetic relationships of the selected isolate (in boldface) with other 16S rDNA sequences of published strains (their accession numbers are present in the brackets). Bootstrap values per 100 bootstrap analyses presented for values greater than 65. The *Delftia acidovorans* strain MBIC1305 (AB020186) was used as out group.

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