ID of Environmentally Relevant Bacteria From Pharmaceutical Industries

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Agenda

• Environmentally relevant bacteria in Pharmaceutical Industries and ID

• Evaluation of the VITEK ® 2 and the new cards for Gram-positive (GP), Gram-negative (GN) and Bacillus (BCL) identification, by using environmental isolates first identified by molecular method

• Conclusion
Introduction

• Identification (ID) of bacteria recovered from the environment of pharmaceutical industries (PI) is of concern.
  – It is a regulatory requirement.
  – It is part of process requirement.
  – It is standard practice in pharma. industries.

• Since isolates in these environments may be stressed, they are known to be sometimes difficult to identify using commercial kit databases (PDA TR #13).
Introduction

- The new VITEK 2 system and related identification cards (GP, GN and BCL) have been designed and released for improved automated identification of bacteria from the environment in the pharmaceutical industries (PI).
- We present an evaluation of this new system when using environmental isolates from PI, previously identified by molecular method (16S rRNA sequencing)
Introduction

• First, we will consider the « Bacterial Domain » to point out the actual diversity of the species we may theoretically face.
• Second, we will present an overview of the main genera and species we did identify in 5 years, by using molecular method, as an expertise lab.
• Third, we will look at the performances of the VITEK 2 system with relevant environmental isolates from PI, previously identified by using nucleic acid based method of comparative sequencing (16S rRNA genes).
• Finally, the results will be discussed regarding the relevance of the species tested, and some comments will be done.
ID needs in Pharmaceutical industries

• In process control
  – Water
  – Raw material bioburden
  – Environmental control and monitoring (EMP)
  – Pre-sterilized bioburden
  – Sterility test
Environmental Monitoring

• Regulations and guidance documents
  – cGMP (USA, EU)
  – CFR (21CFR 211.42, 21 CFR 211.46, 21 CFR 211.22)
  – USP <1116> and EP
  – EMEA (annex I)
  – ISO 14644; ISO 13408; ISO14698-1
  – PDA Technical Report #13
ID requirements in Environmental Monitoring

- ID of environmental isolates is a requirement in many situations.

- Level of identification required (e.g. Group, Genus, Species, sub-Species) varies according to regulations and situations. Ability to set up a database to be able to reveal links in contaminations is a goal.

- It is stressed that environmental isolates are « particular » and that databases of automated systems are sometimes not adapted for them.

- It must be pointed out also that bacterial taxonomy is evolving with the improvement of knowledge, and identification is related to classification.

- Hence it is important to evaluate any automated system with environmental, well identified, isolates to validate how it performs.
Bacterial Domain

- New Classification: Bergey’s 2005
  - Classification criteria
    - Molecular (Nucleic Acid based: Genomic and phylogenetic)
      - Specie definition
        » > 70% DNA-DNA homology and ΔTm < 5°C
        » ≥ 97-98% sequence homology in gene coding for 16S rRNA
        » Multilocus Sequence Typing
    - Phenotypic
      - Morphology, biochemical, physiology (assimilations, fermentation, enzymatic reactions, susceptibilities, etc…)

(Stackebrandt et al., IJSEM 2002)
• Taxonomic resolution of main ID methods

- RFLP
- Ribotyping
- DNA Amplification
- Whole cell proteins
- DNA-DNA Hybridization
- G+C %
- Chimiotaxonomy
- Phenotype
- 16S rRNA Sequencing
- DNA probes
- DNA Sequencing
« Bacterial Domain »

• Species diversity :
  – Archaebacteria :
    • > 259 species on the basis of 16S rRNA gene sequences
  – Eubacteria :
    • > 9021 species on the basis of 16S rRNA gene sequences
      – Gracillicutes (Gram-negative bacteria)
        » Genera : > 538 
        » species : > 2000
      – Firmicutes (Gram-positive bacteria)
        » Genera : > 235 
        » species : > 1346
      – Actinobacteria
        » Genera : > 174 
        » species : > 1534
  – Others
« Bacterial Domain »

• Species diversity : exemples of the impact of molecular taxonomy
  – Eubacteria : new genera and species
    • Gracillicutes (Gram-negative bacteria)
      – *Pseudomonas* : > 8 genera; > 160 species
        » Acidovorax (10), Brevundimonas (12), Burkholderia (35), Comamonas (10), Delftia (2), Pseudomonas (80), Stenotrophomonas (5), Ralstonia (5)
    • Firmicutes (Gram-positive bacteria)
      – *Bacillus* : > 20 genera; > 207 species
        » Alicyclobacillus (5), Amphibacillus (3), Aneurinibacillus (6), Anoxybacillus (9), Bacillus (81), Brevibacillus (11), Filobacillus (2), Geobacillus (23), Gracilibacillus (2), Halobacillus (8), Jeotgallibacillus (2), Lentibacillus (5), Marinibacillus (2), Oceanobacillus (3), Paenibacillus (24), Pontibacillus (2), Sporolactobacillus (5), Virgibacillus (2), Ureibacillus (2), Virgibacillus (10).
« Bacterial Domain »

• Strain diversity :
  – Species definition: up to 30% of DNA-DNA divergence between strains of the same species
    • Genetic variability
    • Phenotypic variability
    • Representativity
      – Type strain of a species
      – Typical strains
      – Atypical strains
      – Environment effect:
        » Selection pressure
        » Stress
        » Sanitizer effects
        » Sterilization process effects (cheat, gaz, radio-sterilization, etc…)
What do we really face?

• Our experience after 5 years of molecular identification…
  – Quantity of bacteria identified : > 3000
    • Gracillicutes (gram negative bacteria) : 38 %
      – Quantity of different species : < 200 ≠ species
    • Firmicutes (gram positive bacteria) : 62 %
      – Quantity of different species : < 300 ≠ species
  • Sources :
    – Environmental monitoring (air-surfaces)
    – Water
    – Raw material, finished products,
    – process, personnel, quality controls
    – Sterility test positives, media-fill test positives, etc …
  • Expert lab…
    – Often difficult to identify isolates…
What do we really face?

• Global Analysis
  – Gram negative Bacteria
    • 70 genera
    • 160 species
What do we really face?

Gram - Genera: Top 20 (78%)

- Achromobacter: 13%
- Acidovorax: 12%
- Acinetobacter: 10%
- Delftia: 7%
- Bradyrhizobium: 6%
- Caulobacter: 13%
- Enterobacter: 12%
- Flavobacterium: 7%
- Methylobacterium: 6%
- Moraxella: 10%
- Pantoea: 12%
- Paracoccus: 7%
- Pseudomonas: 13%
- Pseudoxanthomonas: 6%
- Ralstonia: 7%
- Raoultella: 6%
- Rhodobacter: 10%
- Rosemonas: 7%
- Sphingomonas: 6%
- Stenotrophomonas: 7%

[Pie chart showing the distribution of these genera]
What do we really face?

Gram - Species: Top 35 (60 %)

- Acidovorax temperans
- *Acinetobacter lwoffii*
- *Acinetobacter johnsonii*
- *Bradyrhizobium japonicum*
- *Caulobacter subvibrioides*
- *Enterobacter cancerogenus*
- *Flavobacterium species*
- *Methylobacterium fujiwaraense*
- *Methylobacterium radiotolerans*
- *Moraxella osloensis*
- *Paracoccus yeeii*
- *Porphyrobacter donghaensis*
- *Pseudomonas aeruginosa*
- *Pseudomonas fulgida*
- *Pseudomonas fuscovaginae*
- *Pseudomonas huttensis*
- *Pseudomonas pseudaeruginosa*
- *Pseudomonas rhizosphereae*
- *Pseudomonas saccharophila*
- *Pseudoxanthomonas species*
- *Ralstonia eutropha*
- *Ralstonia insidiosa*
- *Ralstonia pickettii*
- *Raoultella planticola*
- *Rhodobacter massiliensis*
- *Roseomonas mucosa*
- *Sphingomonas alaskaensis*
- *Sphingomonas chlorophenolica*
- *Sphingomonas echinoides*
- *Sphingomonas suberifasciens*
- *Sphingomonas terrae*
- *Sphingomonas xenophaga*
- *Stenotrophomonas maltophilia*
What do we really face?

- Global Analysis
  - Gram Positive Bacteria
    - 80 genera
    - 260 species
What do we really face?

Gram + Genera: Top 10 (85%)

- Bacillus: 40%
- Brevibacterium: 17%
- Micrococcus: 8%
- Mycobacterium: 8%
- Paenibacillus: 8%
- Propionibacterium: 8%
- Staphylococcus: 8%
- Streptomyces: 8%
- Corynebacterium: 8%
- Geobacillus: 8%
What do we really face?

Gram + Species: Top 25 (80%)

- Bacillus cereus groupe
- Bacillus simplex
- Bacillus subtilis groupe
- Bacillus firmus
- Bacillus licheniformis
- Bacillus pumilus
- Bacillus circulans
- Bacillus racemilacticus
- Bacillus megaterium
- Bacillus species
- Brevibacterium casei
- Brevibacterium lutescens
- Micrococcus luteus
- Paenibacillus amylolyticus
- Propionibacterium acnes
- Staphylococcus aureus
- Staphylococcus capitis
- Staphylococcus cohnii
- Staphylococcus epidermidis
- Staphylococcus haemolyticus
- Staphylococcus hominis
- Staphylococcus pasteuri
- Staphylococcus saprophyticus
- Staphylococcus warneri
- Mycobacterium mucogenicum
What do we really face?

Gram + Species: Top 10

- *Staphylococcus aureus*
- *Staphylococcus capitis*
- *Staphylococcus cohnii*
- *Staphylococcus epidermidis*
- *Staphylococcus haemolyticus*
- *Staphylococcus hominis*
- *Staphylococcus pasteuri*
- *Staphylococcus saprophyticus*
- *Staphylococcus warneri*
- *Staphylococcus species*
What do we really face?

Gram + Species: Top 10

- Bacillus cereus groupe
- Bacillus simplex
- Bacillus subtilis groupe
- Bacillus firmus
- Bacillus licheniformis
- Bacillus pumilus
- Bacillus circulans
- Bacillus racemilacticus
- Bacillus megaterium
- Bacillus species
Environmentally relevant bacteria from P.I.

Theoretical variability is extremely high (> 9000 species)
   New species are described almost every day…

Practical variability is not that much high (< 500 species)
   Even for expert lab…

In routine practice one can expect :
   - 80 % of Gram-positive belong to roughly 25 species
   - 60 % of Gram-negative belong to roughly 35 species
Evaluation of the VITEK® 2

- Objectives:
  - Evaluate the performances of the new VITEK®2 system with the GP, GN and BCL cards, for the identification of isolates from pharmaceutical industries.
  - Isolates have been identified by partial 16S rRNA gene sequencing method, considered the « Gold-Standard »
VITEK 2 Compact and the GP, GN and BCL cards

- The VITEK 2 Compact system
Evaluation of the VITEK 2 and *Gram-positive* Card (GP)

- The GP card

  115 taxa claimed, representing 22 genera
Evaluation of the VITEK 2 and Gram-negative Card (GN)

• The GN card

135 taxa claimed, representing 55 genera
  – Enterobacteriaceae
    • 22 genera and 67 taxa
  – Non Enterobacteriaceae
    • 33 genera and 68 taxa
Evaluation of the VITEK 2 and *Bacillus* Card (BCL)

- The BCL card

42 taxa claimed, representing 6 genera

- *Aneurinibacillus* (1), *Bacillus* (18), *Brevibacillus* (8), *Geobacillus* (3), *Paenibacillus* (10) et *Virgibacillus* (2)

* B. anthracis included and identified separately from the other species in *B. cereus* group (*B. cereus*, *B. thuringiensis* and *B. mesenteroides*)
Evaluation of the VITEK 2

• TEST ISOLATES:
  • Environmentally relevant isolates from pharmaceutical industries
    – Originating from different pharmaceutical plants
    – Originating from different types of samples
      • Water analysis, environmental monitoring, sterility test positives, media-fill test positives, bioburden, pre-sterilized bioburden, personnel, etc…
    – In total 404 bacterial strains have been tested
      • 131 GP; 162 GN; 111 BCL
    – Identification of the isolates by using comparative sequencing of the 16S rRNA genes (partial)
      • Sequencing of the 5’end of the 16S rRNA genes (≥ 500 bp) and comparison with databases of sequences of reference strains
      • Microseq v 1.0 and public databases (NCBI)
      • Sequences homologies ≥ 97-98 % with type strains of the species
      • « Gold-Standard »
    – Bacterial species claimed in knowledge databases of GN, GP and BCL cards
VITEK 2 system and GN card

- 162 isolates - 41 taxa
- Performances (Report 114-05NP rev. 1)
  - 92 % of correct identification
  - 8 % of misidentification
  - 0 % of unidentified

<table>
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<tr>
<th>Methode</th>
<th>Total tested</th>
<th>CORRECT ID</th>
<th>One Choice</th>
<th>Low Discrim</th>
<th>Discordant</th>
<th>Unidentified</th>
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<td>VITEK 2 (GN)</td>
<td>162</td>
<td>149</td>
<td>92</td>
<td>131</td>
<td>81</td>
<td>18</td>
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</table>
VITEK 2 system and GN card

• Relevance of the species claimed in database, regarding IDmyk’s expert lab activities:

  • Roughly 60% of the isolates from PI, we did identify in five years, belonged to species (taxa) claimed in the GN card database

  • Improvement:
    – addition of 12 species most frequently encountered for many sites could increase to more than 75% the percentage of isolates that can be identified by the VITEK 2 and GN card system.
VITEK 2 system and GN card

• Comments :

  • *Escherichia coli* and *Escherichia coli* O157H7, *Shigella sp.*
    – *E. coli* and *Shigella dysenteriae* not distinguished by 16S rRNA sequences
    – *E. coli* and *Shigella group* distinguished by VITEK 2
    – *E. coli* and *E. coli* O157H7 distinguished by VITEK 2

  • *Brucella melitensis* and *Ochrobactrum anthropi*
    – Not distinguished by 16S rRNA sequences (≥ 98% homologies)
    – Distinguished by VITEK 2
### VITEK 2 system and GP card

- 131 isolates - 33 taxa
- **Performances** *(Report 114-05NP rev. 1)*
  - 98.5 % of correct identification
  - 1.5 % of misidentification
  - 0 % of unidentified

<table>
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<th>Method</th>
<th>Total Tested</th>
<th>Correct ID</th>
<th>One Choice</th>
<th>Low Discrim</th>
<th>Discordant</th>
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<td>VITEK 2 (GP)</td>
<td>131</td>
<td>129 (98)</td>
<td>120 (92)</td>
<td>9 (6.9)</td>
<td>2 (1.5)</td>
<td>0 (0)</td>
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</tbody>
</table>
VITEK 2 system and GP card

• Relevance of the species claimed in database, regarding IDmyk’s expert lab activities:

  • > 80% of the isolates from PI, we did identify in five years, belong to species (taxa) claimed in the GP card database

  • Improvement: Microbacterium spp., Kocuria spp.
VITEK 2 system and BCL card

• 111 isolates - 17 taxa
• Performances (Report 114-05NP rev. 1)
  – 88.3 % of correct identification
  – 4.5 % of misidentification
  – 7.2 % of unidentified

<table>
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<th>Discordant</th>
<th>Unidentified</th>
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</thead>
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<td>VITEK 2 (BCL)</td>
<td>111</td>
<td>98</td>
<td>88</td>
<td>89</td>
<td>9</td>
<td>5</td>
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VITEK 2 system and BCL card

• Results 1: Claimed Species

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<th>Genus</th>
<th>Species</th>
<th>Total Isolates n=</th>
<th>Correct ID n=</th>
<th>%</th>
<th>One Choice n=</th>
<th>%</th>
<th>Low. Disc. n=</th>
<th>%</th>
<th>Mis. ID n=</th>
<th>%</th>
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<th>%</th>
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<td>Bacillus</td>
<td>amyloliquefaciens</td>
<td>14</td>
<td>14</td>
<td>100%</td>
<td>14</td>
<td>100%</td>
<td>0</td>
<td>0%</td>
<td>0</td>
<td>0%</td>
<td>0</td>
<td>0%</td>
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<td>cereus group</td>
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<td>18</td>
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<td>15</td>
<td>83%</td>
<td>3</td>
<td>17%</td>
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<td>circulans</td>
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<td>2</td>
<td>40%</td>
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<td>0</td>
<td>0%</td>
<td>3</td>
<td>60%</td>
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<td>10</td>
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<td>9</td>
<td>90%</td>
<td>1</td>
<td>10%</td>
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<td>fusiformis</td>
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<td>1</td>
<td>100%</td>
<td>0</td>
<td>0%</td>
<td>1</td>
<td>100%</td>
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<td>0%</td>
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<tr>
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<td>licheniformis</td>
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<td>16</td>
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<td>megaterium</td>
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<td>sporothermodurans</td>
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<td>0%</td>
<td>0</td>
<td>0%</td>
<td>2</td>
<td>100%</td>
</tr>
<tr>
<td>Bacillus</td>
<td>subtilis group</td>
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<td>14</td>
<td>93%</td>
<td>13</td>
<td>87%</td>
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<td>7%</td>
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<td>0%</td>
<td>1</td>
<td>6,7%</td>
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<td>choshinensis</td>
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<td>1</td>
<td>100%</td>
<td>1</td>
<td>100%</td>
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<td>parabrevis</td>
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<td>1</td>
<td>100%</td>
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<td>stearothermophylus</td>
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<td>25%</td>
<td>1</td>
<td>25%</td>
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<td>1</td>
<td>25%</td>
<td>2</td>
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<td>Paenibacillus</td>
<td>amylolyticus/pabuli</td>
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<td>1</td>
<td>50%</td>
<td>0</td>
<td>0%</td>
<td>1</td>
<td>50%</td>
<td>1</td>
<td>50%</td>
<td>0</td>
<td>0,0%</td>
</tr>
<tr>
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<td>glucanolyticus</td>
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<td>67%</td>
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<td>33%</td>
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<td>polymyxa</td>
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<td>1</td>
<td>100%</td>
<td>1</td>
<td>100%</td>
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<td>0%</td>
<td>0</td>
<td>0%</td>
<td>0</td>
<td>0%</td>
</tr>
</tbody>
</table>

| Total             |                      | 111               | 98            | 88,3% | 89            | 80,2% | 9            | 8,1% | 5          | 4,5% | 8        | 7,2% |

In **bold face**, species of the TOP 10 of **Bacillales** species found in routine, from PI, ( > 80 % of total isolates idenfiable)
VITEK 2 system and BCL card

- Relevance of the species claimed in database, regarding IDmyk’s expert lab activities:
  - Roughly more than 15% of *Bacillales* are not identifiable at the species level, even with molecular method of 16S rRNA gene comparative sequencing
    - No reference sequences: species still not described and/or accepted
  - Of the identifiable strains > 80% of the isolates from PI, we did identify in five years, belong to species (taxa) claimed in the GP card database
  - *Improvement*: adding *Bacillus simplex* (mix taxon with *B. badius* or single taxon) to BCL database add 7% to the percentage of environmental isolates from PI covered by BCL card database.
VITEK 2 system and BCL card

• Comments:
  – *Bacillus cereus* group: *B. anthracis*, *B. cereus*, *B. thuringiensis* and *B. mesenteroides*
    • No distinction using 16S rRNA gene sequences (≥ 98% homologies)
      – Phenotype testing (haemolysis), etc…
    • Good distinction using VITEK 2 and BCL card system, especially for *B. anthracis*

  – *Bacillus vallismortis* / *B. subtilis* / *B. atropheus*
    • Species are genotypically similar
      – (*B. vallismortis* sp. nov., a close relative of *B. subtilis*, isolated from soil in death valley california. Roberts et al., IJSB 1996)

  – *Geobacillus stearothermophilus*
    • Take special care for sample preparation and incubation conditions with VITEK 2
Evaluation of the VITEK 2

- **Claimed species global analysis**
  - Gram-negative bacteria
    - 92% of correct identification
    - ID in 3 to 9 hours
  - Gram-positive bacteria (non-sporulating)
    - 98% of correct identification
    - ID in 3 to 7 hours
  - Bacillales
    - 88% of correct identification
    - After 14 hours (end point determination)

**Excellent results with claimed species**
VITEK 2
and relevant environmental isolates from PI

• Overview :
  – This is the first evaluation of an automated identification system using environmental strains from pharmaceutical industries, compared to the reference method of 16S rRNA gene sequencing
  – The evaluation provides validation of the performance of the VITEK 2 with GN, GP and BCL cards
    • The system is accurate, reliable and rapid, even with « tough » environmental isolates
    • Roughly, one can expect that more than 80 % of routine isolates can be exactly identified, rapidly, and in a cost effective manner
  – This evaluation demonstrates how the system is adapted for identification of bacteria found in environment of pharmaceutical industries
  – « Ease of Use » and rapidity of the system have been noted during evaluation
Conclusion

• The VITEK 2 system with the GN, GP and BCL cards identifies environmental isolates from pharmaceutical industries, with accuracies of 88% (BCL), 92% (GN) and 98% (GP) when they correspond to claimed taxa.

• Considering the difficulties routinely encountered to identify these organisms and considering the ease of use of the system, VITEK 2 is a significant advancement for the routine identification of environmental bacteria from pharmaceutical industries.
• Thank-you for your attention