INTRODUCTION:
Diagnosis of the crystal deposition-based arthritic diseases such as gout and pseudogout is dependent on synovial fluid aspirate examination. The currently-used polarized light microscopy (PLM) is not reliably definitive in crystal identification. Raman spectroscopy provides “fingerprint” spectrum of each crystal species and has been shown to be able to identify several pathological crystals. However, there has not been a systematic study of Raman spectroscopy in crystal identification in terms of its detection limit, crystal concentration, and comparison with microscopy technique. Therefore, in the current study we evaluate Raman spectroscopy as a diagnostic tool for identification of arthropathic crystals in synovial aspirates from patients presenting gout-like symptoms.

METHODS:
Collection of synovial fluid: Healthy synovial fluid was obtained from donors with no known history of joint disease from the National Disease Research Interchange (NDRI, Philadelphia, PA). Synovial fluids from symptomatic patients were obtained in a rheumatology clinic under patient consent and institutional review board approval.

Preparation of crystals in healthy synovial fluid: Monosodium urate monohydrate (MSUM) and calcium pyrophosphate (CPPD) crystals were prepared as described earlier. Half mL of healthy synovial fluid was loaded in glass centrifuge tubes (Corning, 8060-15, NY) and 0.3 mg of lyophilized hyaluronidase powder (Sigma, H3506) was added. After 15 min. digestion at room temperature, the solution was centrifuged at 4,000 rpm for 15 min. The supernatant in each tube was discarded. The pellet in each tube was mixed with 0.5 mL of MSUM (or CPPD) solution at 6 different concentrations (1, 2.5, 5, 10, 20, 100 µg/mL). Half mg of lyophilized papain powder (Sigma, P4762) was added to each crystal-containing tube. The above solution was incubated at 37 °C for 30 min and then centrifuged again at 4,000 rpm for 15 min. The supernatant was discarded. The glass tubes which contained the dried crystal pellet were further dried at 50 °C and used for Raman detection.

Detection limit and measurement of crystal concentration by Raman analysis: Thirteen points with predefined coordinates were decided upon the conical bottom of centrifuge tubes which contains the above crystal pellet. The laser was manually focused at these points. MSUM and CPPD crystals were identified by their characteristic peak at 631 cm⁻¹ and 1049 cm⁻¹, respectively. Summation of the intensities of these peaks was taken over the thirteen observation points. The total Raman intensity of each sample was plotted against the known concentration of CPPD or MSUM applied to healthy synovial fluids to find the relationship of each sample was plotted against the known concentration of CPPD or MSUM. The significance of the linear regression was determined by Minitab software (P<0.05).

Preparation of clinical samples for Raman diagnosis: Each clinical sample was divided into two aliquots. One part was sent to Pathology for crystalline identification by PLM and the other part was used for Raman analysis. For each clinical sample, 4 mL of synovial fluid was loaded in centrifuge tubes. Similar hyaluronidase digestion procedure as described in the above was followed, tubes were centrifuged at 4,000 rpm for 15 min. and 3.5 mL of the supernatant was removed, leaving 0.5 mL of liquid. The pellet was re-dispersed in the remaining liquid. Four mg of papain powder and 0.08 g of sodium dodecyl sulfate powder (SDS, Mallinckrodt Baker, Inc., Phillipsburg, NJ) were added and the mixture was kept for 12 hrs at 37 °C. After digestion, the tubes were centrifuged at 4,000 rpm for 15 min., supernatants were removed and the pellet was retained for Raman analysis. Raman analysis of the pellet from the clinical sample was achieved by directly acquiring signals from the birefringent regions as well as by 13-point Raman analysis to quantify the crystal concentration.

RESULTS:
Using current method, it was possible for Raman spectroscopy to detect MSUM and CPPD crystals at concentrations as low as 5 µg/mL and 2.5 µg/mL, respectively (Fig. 1). These detection limits are slightly lower and comparable to that reported for PLM technique (10-100 µg/mL). Raman intensity was linearly related to crystal concentration. The current digestion method was effective in reducing the organics and cell debris in synovial fluid without harming the crystals. Raman diagnosis was in agreement with PLM diagnosis in 32 (25 normal, 6 gout, 1 pseudogout) out of 35 clinical samples (Table 1).

DISCUSSION:
While it seems that there is a general agreement (33 out of 35) between Raman and PLM over the entire sample set, the disagreement in the diseased sample set was 3 out of 10 which is noteworthy. In repeated analyses with the diseased samples, the crystal identification was always in favor of the Raman method. Raman can also give definitive diagnosis of other crystals such as hydroxyapatite, calcium oxalate, and cholesterol since each crystal has characteristic peaks while PLM diagnosis is based on the interference color only. Thirteen observation points provided baseline sufficiency in attaining reasonable variation in concentration measurements. However, the process can be automated in the future to observe greater number of points to further reduce the detection limit and improve crystal identification by Raman spectroscopy. Given that Raman analysis had a good threshold of detection and that its diagnosis is definitive and objective, it needs to be assessed on a larger clinical sample set to derive a refined estimate of sensitivity and specificity by employing the ultimate clinical outcome as the golden standard.

REFERENCES: